Interferon alpha Signaling in Viral Hepatitis

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Table of contents 1

1. Introduction

1.1 The Interferon System

Interferons (IFNs) are broadly expressed cytokines with potent antiviral and growth-inhibitory effects, that constitute the first line of defense against viral infections. The IFN system includes cells that synthesize IFN in response to viral infection and cells that respond to IFN by establishing an antiviral state (*1*). The IFN family includes two main classes of related cytokines: type 1 and type 2 IFNs (*1, 2*). There are many human type 1 IFNs, which share considerable structural homology, including IFNα (which can be further divided into 13 different subtypes), IFNβ, IFNε, IFNκ and IFNω (*1, 2*). The genes encoding human type 1 IFNs are clustered on chromosome 9 (*2*). The common cell-surface receptor on which all type 1 IFNs bind is known as the type 1 IFN receptor (*1, 2*). The type 1 IFN receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with the Janus activated kinases (JAKs), JAK1 and TYK2 (Fig.1). In contrast to the type 1 IFNs, that are all clustered on chromosome 9, there is one type 2 IFN, IFNγ (*1, 2*) that is located on human chromosome 12. There is no marked structural homology between type 1 and 2 IFNs (*1-4*). The receptor which IFNγ binds to is known as the type 2 IFN receptor (*5*). This receptor is also composed of two subunits called IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2 (Fig1). Recently a new class of IFNs has emerged, the IFNλ 1,2 and 3, also known as interleukin 29, 28A and 28B. They display antiviral properties, but they are distinct from the type 1 and type 2 IFNs and bind to a different receptor, which is composed of two chains, IFNLR1 and IL-10Rb (*6*). The initial step in both type 1 and type 2 IFN mediated signaling is the activation of the receptor associated JAKs by dimerization of the receptor subunits, followed by autophosphorylation and activation of the associated JAKs. JAKs then activate the classical JAK-STAT (signal transducer and activator of transcription) - signaling pathways (Fig.1). But they also activate directly or indirectly several other downstream cascades.

1.2 The Jak-STAT signaling pathway

1.2.1 Activation of the Jak-STAT pathway

The first signaling pathway shown to be activated by IFNs was the JAK-STAT pathway. Discovered in the 1990s (*7-10*), this pathway provides a simple model for IFN-mediated signaling (Fig.1). The model involves rapid nuclear translocation and initiation of gene transcription by STATs that have been activated at the plasma membrane in response to JAK-mediated phosphorylation. This mechanism is required for the induction of many of the effects of IFNs. The binding of IFNα or other type 1 IFNs to the type 1 IFN receptor results in the rapid autophosphorylation and activation of the receptor associated JAKs TYK2 and JAK1 (*10*), which in turn regulate the phosphorylation and activation of STATs (*11, 12*). STAT 1,2,3 and 5 are activated in response to type 1 IFNs (*11-14*). After phosphorylation by JAKs, the activated STATs form homo- or heterodimers that translocate to the nucleus to initiate transcription by binding specific sites in the promoters of IFN-stimulated genes (ISGs), that mediate various biological responses (*3, 11, 12*). There are two different binding elements in the promoters of IFN-stimulated genes: IFN-stimulated response elements (ISREs) and IFN-γ-activated site (GAS) (*4, 12, 15*). The complex, which binds to the ISRE is composed of the phosphorylated forms of STAT1 and STAT2, together with IRF9, which does not undergo tyrosine phosphorylation (11-13). Other STAT complexes that are induced by type 1 IFN, including homodimers like STAT1-STAT1 or heterodimers like STAT1-STAT3, bind to the GAS element (*4, 12, 13*). Of the hundreds of known IFN-stimulated genes, some have only ISREs or only GAS elements, whereas others have both elements in their promoters (Fig.1).

The transcription of type 2 IFN (IFN-γ)-dependent genes is regulated by GAS elements. After binding of IFNγ to the type 2 receptor, JAK1 and JAK2 are activated and regulate downstream phosphorylation of STAT1 on the tyrosine residue at position 701. The phosphorylated STAT1 molecules form STAT1-STAT1 homodimers, which translocate to the nucleus and bind to GAS elements to induce transcription (*16*). There is no formation of ISGF3 complexes in response to IFNγ and therefore no induction of genes that have only ISREs in their promoter (Fig.1).

1.2.2 Negative Regulation of the Jak-STAT Pathway

Negative regulation of the Jak-STAT pathway occurs at two different points in the signaling cascade: at the level of the Janus kinases and at the level of STATs. The suppressor of cytokine signaling (SOCS) family members, SOCS1 and SOCS3, inhibit the catalytic activity of Janus kinases and therefore they prevent the phosphorylation and activation of IFNα induced STATs (*17*). A second inhibition occurs downstream of STAT1 activation by protein inhibitor of activated STAT1 (PIAS1). PIAS1 binds to STAT1 dimers and therefore prevents the binding of STAT1 dimers to the response elements in the promoter of target genes (*18, 19*). The binding of PIAS1 to STAT1 is regulated by methylation of STAT1 by protein arginine methyl transferase 1 (PRMT1) (*20*). Arginine methylation inhibits binding of PIAS1 to STAT1, whereas

hypomethylation of STAT1 enhances its association with PIAS1 and therefore prevents the binding of activated STAT1 to DNA. Recently it was shown that PIAS1 selectively regulates a subset of IFNγ- or IFNβ-inducible genes by interfering with the recruitment of STAT1 to the gene promoter. The DNA-binding affinity of STAT-binding sites present in the promoters of STAT1 target genes can influence the PIAS1 effect. PIAS1 has a more profound effect on genes containing weak STAT-binding sites (for example, Gbp1, Ly6e), than genes containing strong STAT1-binding site (for example, Irf1)(*19*).

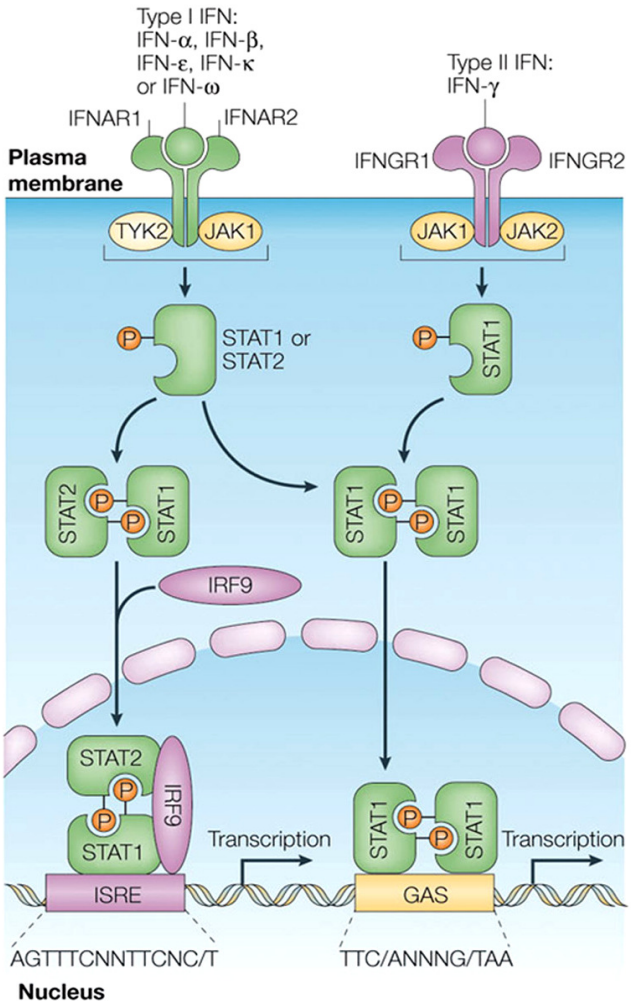

Fig.1: Interferon receptors and activation of JAK-STAT pathways by type 1 and type 2 interferons (*21*).

1.2.3 Viral Interference with the Jak-STAT pathway

Both DNA and RNA viruses encode proteins that impair the activity of the Jak-STAT signaling pathway to inhibit the induction of an antiviral response. Multiple mechanisms appear to be involved in this inhibition. For example, poxviruses encode soluble IFN receptor homologues. These proteins are secreted from poxvirus-infected cells and bind IFNs, thereby preventing them from acting through their natural receptors to elicit an antiviral response (*22*). The E1A protein of the adenovirus inhibits the DNA binding activity of ISGF-3 (*23*). Also the sendai virus, a paramyxovirus virus, that replicates in the cytoplasm of the host cell, inhibits the IFN-induced antiviral response by interfering with the transcriptional activation of IFN-inducible genes (*24*) to prevent the establishment of an antiviral state. The herpesvirus varicella-zoster virus (VZV) inhibits the expression of STAT-1 and Jak-2 (*25*) and another herpesvirus, the cytomegalovirus (CMV) inhibits the IFN signaling by decreasing the level of Jak-1 due to enhanced protein degradation (*26*). Several RNA viruses encode gene products that inhibit Jak-STAT signaling. For example, infection with simian virus 5 or mumps virus leads to an increased proteosome-mediated degradation of STAT1 (*27*), whereas infection with parainfluenza virus type 2 leads to degradation of STAT-2 (*28*). The hepatitis C virus inhibits the IFN signaling by blocking methylation of STAT1. The expression of HCV proteins in liver cells of transgenic mice reduced STAT1 methylation and consequently increased association of STAT1 with PIAS1. Importantly, reduced methylation and increased PIAS1-STAT1 binding was also observed in liver biopsies from patients with chronic hepatitis C. There is evidence that protein phosphatase 2A is involved in mediating this effect by inhibiting the methylation of STAT1 through PRMT1: first, PP2A was found to be overexpressed in HCV transgenic mice and in liver biopsies from patients with chronic hepatitis C; second, Huh7 cell stably expressing the catalytically active HA-PP2Ac have hypomethylated STAT1 in the absence of any HCV proteins; and third, inhibition of PP2A with okadaic acid partially restores methylation of STAT1 (*29, 30*).

1.3 Viral hepatitis

1.3.1 Hepatitis C

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) worldwide. Currently about 170 million people world wide (3%) are infected with HCV. Acute hepatitis C is usually asymptomatic but leads to chronic infection in 50-80% of cases. Patients with chronic HCV are at high risk to develop liver cirrhosis and HCC. The mechanisms underlying viral persistence and pathogenesis are poorly understood. So far no protective vaccine is available and therapeutic options are limited. The currently used therapy is pegylated interferon alpha (PEG-IFNα) combined with ribavirin. Only approximately 50% of treated patients develop a sustained virologic response (SVR, no HCV detectable in the blood six months after the end of the treatment) after treatment. The cause of treatment failure in non-responders is not fully understood, but recently viral interference with the Jak-STAT pathway has emerged as possible cause (*29, 30*).

HCV was identified more than a decade ago (*31*). Investigation of the replication cycle has been limited by the low viral titers found in sera and livers of infected individuals and the lack of an efficient cell culture system or small animal model permissive for HCV. However, considerable progress has been made using heterologous expression systems, functional cDNA clones (*32*) and subgenomic replicon systems (*33*). These systems use self-replicating subgenomic viral RNAs. But replicons only recapitulate the intracellular life cycle of HCV, they do not produce infectious virus. However, an HCV isolate from a Japanese patient with fulminant HCV infection could be identified, that, for unknown reasons, replicates in a human hepatoma cell line (*34, 35*) and cells releases virus particles that are infectious. This *in vitro* system provides new possibilities to study the HCV life cycle and to develop novel antiviral drugs.

HCV belongs to the genus Hepacivirus within the Flaviviridae family. The 9.6 kb plus-strand RNA genome contains a 5` noncoding region (5`NCR), a long open reading frame encoding a polyprotein precursor of about 3000 amino acids, and a 3`NCR. The 5`NCR is highly conserved and contains an internal ribosomal entry site (IRES), that is essential for cap-independent translation of viral RNA (*36*). The polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases into structural and non-structural proteins (Fig.2). The structural proteins include the core protein that forms the viral nucleocapsid and the envelope glycoproteins E1 and E2 (*37*). The nonstructural proteins include the NS2-3 autoprotease responsible for the cleavage of the polyprotein precursor at the NS2/NS3 junction, the NS3 serine protease (a NTPase/RNA helicase), the NS4A, that function as a cofactor for the NS3 serine protease, the NS4B involved in the formation of the membranous web, the NS5A and the NS5B RNA-dependent RNA polymerase (RdRp), the key enzyme responsible for replication (*38-41*).

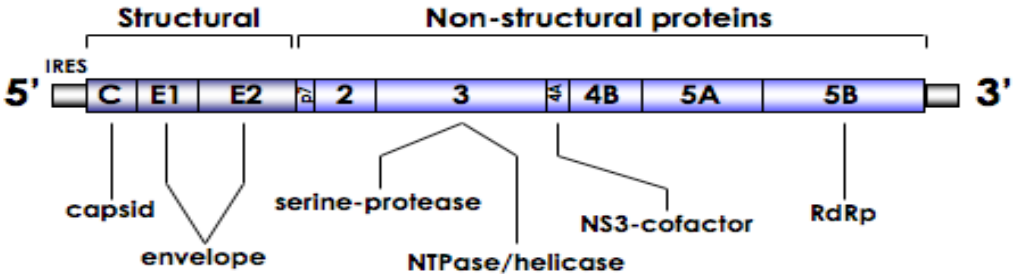

Fig. 2: Schematic representation of the HCV genome with the sructural proteins C, E1 and E2 and the non-structural proteins (*42*). Core (C) forms the nucleocapsid, E1 and E2 are the envelope glycoproteins, p7 probably forms an ion channnel involved in some step of virus production; NS2 is a cysteine protease, NS3 forms a stable comples with NS4A creating the viral serine-type protease whereas the C terminal domain contains nucleotide triphosphate (NTPase) and helicase activity, NS4B can induce the formation of intracellular membrane vesicles presumably forming the scaffold of the viral replication complex; phosphoprotein NS5A is a RNA-binding replication factor; and NS5B is the RNA-dependent RNA polymerase (RdRp).

The life cycle of HCV starts with the binding to cell surface receptor CD81(*43*) on the membrane of the hepatocyte and with internalization into the host cell. After this first initial step, the cytoplasmic release and uncoating of the viral RNA genome follows.

Afterwards IRES-mediated translation, polyprotein processing and RNA replication start. The last steps are packaging, assembly and virion maturation, followed by virion release from the host cell (*44*)

1.3.2 Hepatitis B

Hepatitis B virus (HBV) is a member of the Hepadnaviridae (hepatotropic DNA virus) family. Hepadnaviruses display a strong preference for infecting liver cells, but small amounts of viral DNA can also be found in kidney, pancreas and monocuclear cells (*45, 46*). HBV virions are double-shelled particles containing an outer lipoprotein envelope composed of three related envelope glycoproteins (or surface antigens) and within the envelope the viral nucleocapsid or core (*47*). The core contains the viral genome, a relaxed-circular, partially duplex DNA of 3.2 kb and a polymerase, responsible for the synthesis of viral DNA in infected cells (*48*). Additionally to virions, cells infected with HBV produce filamentous and spherical particles. These HBsAg particles contain only envelope glycoproteins and host-derived lipids (*49, 50*). The HBV genome consists of four open reading frames. The presurface-surface region encodes the three viral surface antigens (S protein known as HBsAg, M protein and L protein) by differential initiation of translation (*47, 49-51*). The preC-C region encodes the hepatitis B core antigen (HBcAg) and the hepatitis B e antigen (HBeAg)(*47*). HBeAg is secreted into the blood (*52*), and so far its function is unclear. The P coding region encodes the viral polymerase, an enzyme involved in DNA synthesis and RNA encapsidation. Finally the X protein (HBx) is transcribed from the X open reading frame. This protein is required for the *in vivo* replication and for the spread of the virus (*53*).

The viral replication cycle starts with the binding of HBV virions to cell-surface receptors, followed by membrane fusion. The free core is then transported across the cytosol to the nucleus. There the HBV genome is converted to a covalently closed circular form (cccDNA) (*54*), serving as transcriptional template for host RNA polymerase 2. Afterwards the viral RNA is translocated to the cytoplasm, where its translation yields the viral proteins. The following step is the assembly of the nucleocapsids in the cytosol. During this process a single molecule of genomic RNA is incorporated into the viral core and the reverse transcription starts (*55, 56*). Most of the cores, bearing the mature genome acquire lipoprotein envelopes containing the viral L, M and S surface antigens and are then exported from the cell. The HBV replication cycle is not directly cytotoxic to cells, but the host immune responses to viral antigens displayed on infected hepatocytes are the principal cause of liver damage (*57*).

Primary HBV infection in nonimmune hosts can be either symptomatic or asymptomatic. Most of the primary infections in adults are self-limited, with clearance of the virus from blood and liver (*58, 59*). However some primary infections in healthy adults (less than 10 percent) develop into persistent infections, which can be symptomatic or asymptomatic. Patients with normal serum aminotransferase levels and normal findings on liver biopsy are called asymptomatic chronic HBV carriers. Those patients with abnormal liver function tests and pathological findings on liver biopsy have chronic hepatitis B (CHB). Worldwide more than 350 million people are chronically infected with HBV (*60, 61*). These patients are at risk to develop liver cirrhosis and HCC. Approved treatments for

chronic hepatitis B include nucleos(t)ide analogues like lamivudine and adefovir, or interferon α (IFNα), recently in pegylated form (PegIFNα) (*61*). However, over 60 percent of patients do not respond to the PegIFNα treatment and continue to suffer from chronic active infection. The molecular mechanisms causing the ineffectiveness of IFNα treatment in chronic hepatitis B are not known so far.

1.4 Structure and function of protein phosphatase 2A

Reversible protein phosphorylation is an essential regulatory mechanism in many cellular processes, and cells use this post-translational modification to change the properties (activity, cellular localization, etc.) of proteins involved in specific pathways. One important protein phosphatase in cells is the protein phosphatase type 2A (PP2A). PP2A accounts for as much as 1 percent of total cellular proteins and for the major portion of serine/threonine phosphatase activity in most tissue and cells (*62*). Deletion of the gene-encoding PP2A catalytic subunit is lethal in mice, showing the essential role of PP2A (*63*). PP2A dephosphorylates a myriad of substrates, and is involved in the regulation of many cellular activities including cell cycle regulation, cell morphology, development, signal transduction, translation, apoptosis, and stress response (*64*). An approximately 36 kD catalytic subunit (PP2Ac), that is associated with a 65 kD scaffolding subunit called A or PR65, which modulates the enzymatic activity of the catalytic subunit, build the core enzyme of PP2A (*65*). Distinct classes of regulatory B subunits can bind to AC to form a wide variety of heterotrimeric complexes (Fig.3). The expression of PP2A is tightly regulated (*66*), but downregulation of PP2Ac has been found during all-trans-retinoic acid induced differentiation of HL-60 cells (*67*) or during peroxisome proliferator-activated receptor-γ induced adipocyte differentiation (*68*). On the other side, upregulation of PP2Ac was found in response to colony-stimulating factor 1 (*69*). Recently it was shown, that the expression of HCV proteins in livers of transgenic mice induce an upregulation of PP2Ac, that was also observed in liver biopsies from patients with chronic HCV infection(*30*).

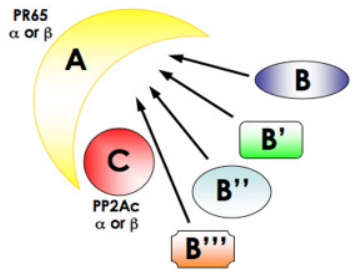

Fig.3: Structure of PP2A holoenzyme.
The core enzyme is a dimer, consisting of the 36kDa catalytic subunit (C) and the regulatory subunit of molecular mass 65kDa, termed PR65 or A subunit (A). A and C are encoded by two genes (α and β). A third regulatory B subunit can be associated with this core structure. At present, four different families of B subunits (B, B`, B`` and B```) have been identified (*64*).

1.5 Protein Arginine Methyltransferase 1 (PRMT1)

There is strong evidence that PP2A inhibits PRMT1 and therefore leads to hypomethylation of STAT1. Methylation of STAT1 displays the third important posttranslational modification beside tyrosine and serine phosphorylation that regulates STAT1 mediated transcriptional activation of IFNα target genes. Methylation of arginine 31 of STAT1 is catalyzed by PRMT1 and inhibits the binding of PIAS1 to STAT1 (*20*). PRMT1 is a ubiquitously expressed protein arginine methyltransferase type 1. The type 1 enzymes recognize substrates containing GAR motifs (glycine and arginine-rich motifs), and the methylation occurs in two steps with an intermediate monoarginine, followed by an asymmetric dimethylarginine. Substrates of PRMT1are involved in transcriptional regulation, signal transduction and DNA repair (*70*). Methylation of histone H4 through PRMT1 for example leads to a change in chromatin conformation and therefore to a change in transcription. Interestingly, PRMT1 is also responsible for arginine methylation of HCV helicase NS3 on arginine 1493 (*71*). Furthermore mutational analysis showed, that this arginine together with arginine 1490 is essential for the enzymatic activity of NS3 helicase (*72*). However, the consequences of this possttranslational modification of NS3 and the possible effect of PP2A on the methylation of NS3 have not been investigated.

1.6 The ER stress response

The endoplasmatic reticulum (ER) is the first organell in the secretory pathway responsible for the synthesis, modification and delivery of proteins to their target sites. In the ER, proteins fold into their native conformation and undergo post-translational modifications like asparagine-linked glycosylation and the formation of intra-or intermolecular disulfid bonds (*73-75*). Only correctly folded proteins are exported to the Golgi complex, while malfolded proteins are targeted for degradation (*76*). Folding of proteins requires a complex ER-resident protein folding machinery consisting of three classes of proteins: foldases, molecular chaperones and the lectins calnexin, calreticulin and ER degradation-enhancing α-mannosidase-like protein (EDEM). Foldases are enzymes that catalyze steps in protein folding (*77, 78*). Molecular chaperones facilitate protein folding by shielding unfolded regions from surrounding proteins without enhancing the rate of protein folding. One prominent chaperone is BiP (binding protein or glucose regulated protein GRP78). The lectins play an important role in the quality-control machinery of the ER (*79, 80*). In several situations the demand on the ER-resident protein folding machinery exceeds its capacity, e.g. during viral infection (*81, 82*). As a result the ER is overloaded with native, unfolded proteins, which induces an ER stress response, called the unfolded protein response (UPR) (*83*). There are two adaptive mechanisms to bring the folding capacity of the ER to its normal physiological state: increasing the folding capacity through induction of ER-resident molecular chaperones and foldases and decreasing the biosynthetic load of the ER through down-regulation of

protein synthesis on a transcriptional and translational level (*84, 85*) as well as increased clearance of unfolded proteins from the ER through ER associated degradation (ERAD)(*86, 87*). If these mechanisms do not solve the stress situation, apoptosis is initiated in higher eukaryotic organisms to eliminate unhealthy or infected cells (*88*). Three transmembrane proteins transduce the unfolded protein signal across the endoplasmic reticulum membrane: inositol requiring 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (Fig.4). In an inactive state the luminal domains of these three proteins are associated with BiP (*89, 90*). During ER stress, BiP is competitively titrated from the luminal domains by the huge excess of unfolded proteins in the ER lumen, resulting in activation of these proximal signal transducers.

ATF6 translocates to the Golgi complex after being released from BiP and is cleaved in the Golgi by site-1 and site-2 proteases (S1P, S2P). These proteolytic reactions release the cytosolic N-terminal portion of ATF6 encoding a basic leucine zipper (bZIP) transcription factor (*91-93*). ATF6 binds to the ATF/CRE element (*94*) and to the ER stress response elements 1 and 2 (ERSE-1/2)(*95, 96*). Important targets regulated by ATF6 are BiP/GRP78, XBP-1 and CHOP (*96*) (Fig.4).

The IRE1 pathway starts with IRE1 oligomerization after dissociation from BiP, followed by activation of the RNase domain of IRE1 through autophosphorylation. The substrate for the endoribonuclease is the mRNA for the bZIP transcription factor XBP-1 (*97-99*). XBP-1 splicing introduces a frame-shift and an alternative C-terminus with increased transcriptional activation potential. XBP-1 controls a subset of ER-resident molecular chaperones 149 (*100*).

PERK is activated by release of BiP from its ER luminal domain. PERK then oligomerizes and phosphorylates its substrate eIF2α. Phosphorylation of eIF2α by PERK shuts-off general translation (29). During inhibition of translation, short-lived proteins like cyclin D1 are cleared from the cell. Loss of cyclin D1 during ER stress arrests mammalian cells in G1-phase (*101*). However the translation of proteins is not completely inhibited. There are some selected mRNAs, which are preferentially translated. One of these mRNAs is the mRNA of ATF4 (*102*), which regulates as a transcription factor the transcription of CHOP and GADD34 (*103*).

Another important function of the ER beside the synthesis, modification and delivery of proteins to their target sites is the storage of Ca^{2+}. In mammalian cells the ER is the major site for Ca^{2+} storage. ER luminal Ca^{2+} concentrations reach 5 mM, compared to 0.1 mM in the cytosol (*105*). The majority of the ER-resident molecular chaperones and foldases are vigorous Ca^{2+} binding proteins. Any change of the ER Ca^{2+} pool affects the folding capacity and activity of these enzymes (*106*).

It is well known that viral infection of a cell can induce ER stress. As a processing plant for folding and post-translational modifications of proteins, the ER is an essential organelle for viral replication and maturation. In the course of productive infection, a large amount of viral proteins are synthesized in infected cells, where unfolded or misfolded proteins activate the ER stress response. For HCV it has been published that the expression of envelope E1 and E2 proteins (*107, 108*), the expression of HCV core proteins (*109*) or the transfection of subgenomic replicons (*110*) induce an ER stress response. But there are also publications reporting that HCV disrupts part of the ER stress response (*111, 112*)

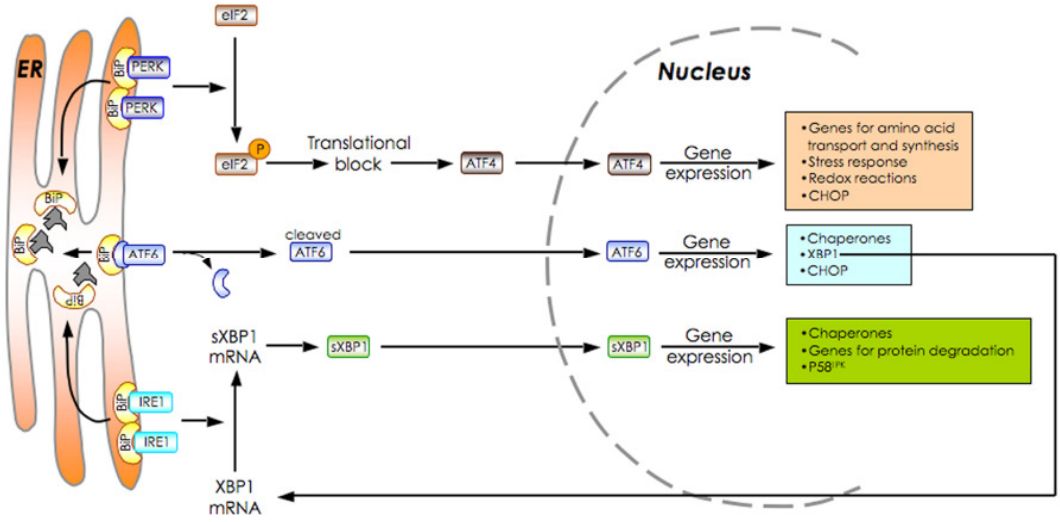

Fig.4: The unfolded protein response.
Upon aggregation of unfolded proteins, BiP dissociates from the three endoplasmic reticulum (ER) stress receptors, pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), allowing their activation. The activation of the receptors occurs sequentially, with PERK being the first, rapidly followed by ATF6, whereas IRE1 is activated last (*104*). The phosphorylation of eIF2 by PERK leads to a general inhibition of translation, but selected mRNAs, like the transcription factor ATF4 mRNA, are more efficiently translated. ATF4 initiate the expression of a subset of ER stress response genes. After translocation to the Golgi and cleaveage, ATF6 function as an active transcription factor and induces also ER stress response genes. Activated IRE1 splices the cytoplasmic XBP1 mRNA, and an active transcription factor is translated from this spliced mRNA.

2. Aims of the study

The overall aim of the studies presented in this thesis, was to investigate the molecular mechanisms induced by HCV respectively HBV to inhibit IFNα signaling in cells

2.1 Aim 1

The first project investigated the functional consequences of NS3 methylation, the HCV helicase. Furthermore, the regulation of PRMT1 by PP2A was studied, because PRMT1 is responsible for NS3 methylation and PP2A is upregulated by HCV.

2.2 Aim 2

Based on our current model of HCV interference with IFNα signaling (Fig.5), the second project explored if the HCV induced STAT1 hypomethylation could be corrected by treatment of cells with the methylgroup donor AdoMet and betaine.

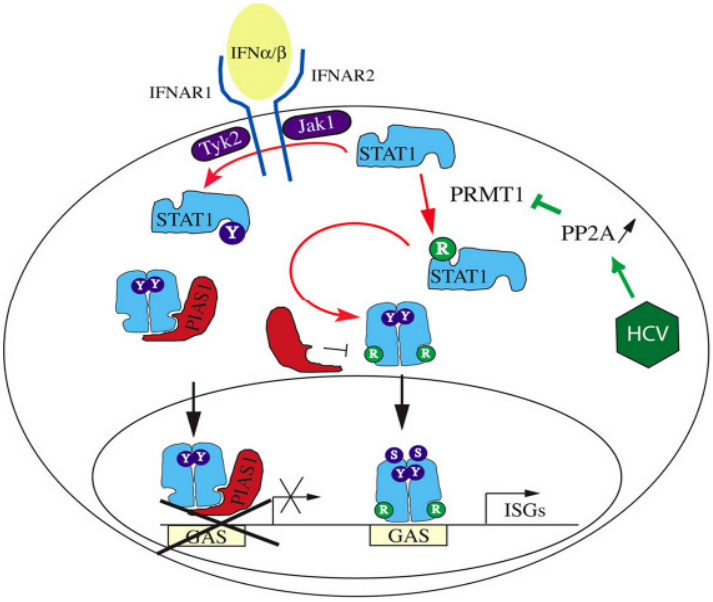

Fig.5: Interference of HCV with IFNα signaling: current working model.
The expression of HCV proteins induces the upregulation of PP2A. Increased PP2A levels inhibit the activity of PRMT1, leading to hypomethylation of STAT1. The hypomethylated STAT1 is bound by PIAS1 and therefore STAT1 dimers, even when phosphorylated upon IFNα treatment, cannot bind anymore to the promoter of IFN target genes.

2.3 Aim 3

The third aim was to investigate the IFNα signaling in HBV infection. As mentioned above, only 40% of chronic hepatitis B patients treated with IFNα shows clearance of the virus, and it is still unknown what mechanisms are involved in HBV resistance to IFNα treatment. Despite the fact, that HBV and HCV are completely unrelated viruses, we hypothesize that to establish resistant infection HBV could inhibit IFNα signaling using similar mechanisms than HCV.

2.4 Aim 4

The fourth project investigated the molecular mechanisms of PP2A upregulation in cells expressing HCV and HBV proteins.

3. Results

3.1 Upregulation of Protein Phosphatase 2Ac by Hepatitis C Virus Modulates NS3 Helicase Activity through Inhibition of Protein Arginine Methyltransferase 1

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Upregulation of Protein Phosphatase 2Ac by Hepatitis C Virus Modulates NS3 Helicase Activity through Inhibition of Protein Arginine Methyltransferase 1

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Hepatitis C virus (HCV) is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma worldwide. HCV has a positive-strand RNA genome of about 9.4 kb in size, which serves as a template for replication and for translation of a polyprotein of about 3,000 amino acids. The polyprotein is cleaved co- and posttranslationally by cellular and viral proteases into at least 10 different mature proteins. One of these proteins, nonstructural protein 3 (NS3), has serine protease and NTPase/RNA helicase activity. Arginine 467 in the helicase domain of NS3 (arginine 1493 in the polyprotein) can be methylated by protein arginine methyltransferase 1 (PRMT1). Here we report that the methylation of NS3 inhibits the enzymatic activity of the helicase. Furthermore, we found that PRMT1 activity itself is regulated by protein phosphatase 2A (PP2A). PP2A inhibits PRMT1 enzymatic activity and therefore increases the helicase activity of NS3. This is important, because we found an increased expression of PP2A in cell lines with inducible HCV protein expression, in transgenic mice expressing HCV proteins in hepatocytes, and in liver biopsy samples from patients with chronic hepatitis C. Interestingly, up-regulation of PP2A not only modulates the enzymatic activity of an important viral protein, NS3 helicase, but also interferes with the cellular defense against viruses by inhibiting interferon-induced signaling through signal transducer and activator of transcription 1 (STAT1). We conclude that up-regulation of PP2A might be crucial for the efficient replication of HCV and propose PP2A as a potential target for anti-HCV treatment strategies.

Infections with hepatitis C virus (HCV) become chronic in most patients, and chronic hepatitis C (CHC) can progress to cirrhosis and hepatocellular carcinoma (9, 17, 23, 29). To persist in the host, the virus has to evade the immune system. We have shown previously that HCV interferes with alpha interferon (IFN-α)-induced signaling through the Jak-STAT pathway (5, 10, 14). The interferon system is an important component of the host response against viruses, and mice with deficiencies of IFN receptors or of signal transducer and activator of transcription 1 (STAT1) are highly susceptible to viral infections (2, 11, 26). IFN-α/β binding to its receptor activates members of the Jak family of tyrosine kinases, which then phosphorylate STAT1, STAT2, and STAT3 on a single tyrosine residue. Phosphorylated STATs form dimers, translocate into the nucleus, bind to promoter elements of interferon-stimulated genes, and activate the transcription of interferon-stimulated genes (7). This activation cycle is terminated by tyrosine dephosphorylation in the nucleus, followed by the decay of dimers and the nuclear export of STATs (8, 35). The pathway is tightly controlled by a number of inhibitory proteins (20, 33), among them protein inhibitor of activated STAT1 (PIAS1) (25). PIAS1 inhibits the last step in the Jak-STAT pathway, i.e., DNA binding. The complex formation between STAT1 and PIAS1 is regulated by an important posttranslational modification of STAT1, arginine methylation (30). Methylation of STAT1 is catalyzed by protein arginine methyltransferase 1 (PRMT1) and protects STAT1 from binding and inactivation by PIAS1 (30).

We have previously reported that HCV inhibits IFN-α-induced signaling at the level of STAT DNA binding (5, 14). Further analysis of the mechanism responsible for HCV interference with STAT signaling led to two a priori independent observations made both with extracts from liver cells of HCV transgenic mice and with liver biopsy samples from patients with CHC: (i) STAT1 was hypomethylated and bound by PIAS1, and (i) protein phosphatase 2Ac (PP2Ac) expression was increased (10). PP2A is a heterotrimeric protein phosphatase consisting of a 36-kilodalton catalytic C subunit (PP2Ac), a 65-kilodalton structural A subunit, and a variable regulatory B subunit. PP2A is expressed in all cell types, is primarily a serine/threonine phosphatase, and is involved in a wide range of cellular processes, including cell cycle regulation, cell morphology, development, signal transduction, translation, apoptosis, and stress response (15, 27). An involvement of PP2A in the regulation of PRMT1 has not been reported before. However, we found that expression of a constitutively active form of PP2Ac in human hepatoma cells (Huh7) resulted in hypomethylation of STAT1 and inhibition of IFN-α-induced signaling (10), demonstrating that PP2Ac is upstream in a signaling

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pathway that regulates STAT1 methylation. We therefore further investigated the role of PP2A in the regulation of STAT1 methylation. In the present paper, we report that PP2Ac interacts directly with PRMT1 and that it inhibits the enzymatic activity of PRMT1.

Interestingly, PRMT1 is also responsible for arginine methylation of NS3, a nonstructural HCV protein with protease and helicase activity (31). The NS3 helicase-NTPase domain consists of the 442 C-terminal amino acids of NS3 and belongs to the DEAD (Asp-Glu-Ala-Asp) box RNA helicase family (19, 21, 24). The domain has probably multiple functions, including RNA-stimulated NTPase activity, RNA binding, and unwinding of RNA regions with extensive secondary structure. Mutational analysis of NS3 helicase revealed that arginines 1490 and 1493 (position in the polyprotein) are essential for enzymatic activity (16). Interestingly, arginine 1493 (arginine 467 of the helicase domain) has been shown to be posttranslationally modified by methylation through PRMT1 (31). However, the functional consequence of this modification is not known. Here we show that arginine methylation inhibits the enzymatic activity of NS3. Furthermore, we show that PRMT1 itself is negatively regulated by PP2A. Therefore, by increasing the expression level of PP2Ac, HCV can indirectly regulate the helicase activity of NS3.

Taken together, our results support an important role of PP2A in the regulation of the viral life cycle of HCV. By inducing PP2Ac overexpression, HCV achieves both an inhibition of IFN-α signaling and an increase in NS3 helicase activity.

MATERIALS AND METHODS

Reagents, antibodies, and plasmids. Human IFN-α was obtained from Hoffmann LaRoche (Basel, Switzerland). Purified PP2Ac and okadaic acid (OA) were purchased from Upstate (LucernaChem, Luzern, Switzerland) and Sigma (Fluka Chemie GmbH, Buchs, Switzerland), respectively. His-Bax was purchased from Santa Cruz (LabForce AG, Nunningen, Switzerland). Glutathione *S*-transferase (GST) columns (MicroSpin GST purification module), IPTG (isopropyl-β-D-thiogalactopyranoside), and ^{3}H-AdoMet (specific activity, 15 Ci/mmol) were obtained from Amersham Biosciences (Amersham Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland). pGEX-HRMT1L2 (human PRMT1) was a generous gift from Pamela A. Silver. The NS3 helicase domain (NS3h) construct His^{5n}-Hel-His^{5c} was a gift from David Frick. It contains amino acids 166 to 631 of NS3 (amino acids 1197 to 1663 of the polyprotein) and is derived from the genotype 1a isolate H77 (accession number, AAB66324). A detailed description of the construct has been published (13).

For protein expression, transfected bacteria cells were grown overnight in LB medium supplemented with ampicillin or kanamycin until they reached an optical density of 0.6 to 0.7. GST-PRMT1 expression was induced with 1 mM IPTG for 3 h at 30°C. NS3h expression was induced with 1 mM IPTG for 3 h at 37°C. After lysis of the bacteria, the fusion proteins were purified with a GST MicroSpin column (Amersham) or a Ni-nitrilotriacetic acid spin kit (QIAGEN AG, Basel, Switzerland) according to the manufacturers' instructions. Purified NS3h was dialyzed using a Float-A-Lyzer system (Socochim, Lausanne, Switzerland). Purified proteins were then concentrated and buffers exchanged in a storage buffer (50 mM NaCl, 50 mM Tris, 1 mM EDTA) with iCON concentrator (Pierce).

Cell lines. The generation of HA-PP2Ac cells was described previously (10). The cells were cultured in 10% calf serum/minimum essential medium supplemented with neomycin (Invitrogen, Basel, Switzerland). U-2 OS human osteosarcoma-derived, tetracycline (tet)-regulated cell lines UHCV-57.3 and UHCV-32, which inducibly express the entire HCV polyprotein derived from an HCV H consensus cDNA (18, 28), and UGFP-20 cells, which inducibly express green fluorescent protein, have been described previously (32).

Preparation of extracts from cells. Cell extracts for Western blots and electrophoretic mobility shift assays were prepared as described previously (10).

RNA isolation, reverse transcription, and SYBR-PCR. Total RNA was isolated from the cells using a Perfect RNA Eukaryotic Mini kit (Eppendorf, Vaudaux-Eppendorf, Basel, Switzerland) according to the manufacturer's instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega, Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Promega) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70°C and then for 1 h at 37°C. The reaction was stopped by heating to 95°C for 5 min. SYBR-PCR was performed based on SYBR green fluorescence (SYBR green PCR master mix, Applied Biosystems, Foster City, CA). To prevent genomic DNA amplification, the primers for RPL19 and PP2Ac were designed across exon-intron junctions. The primers for RPL19 were 5′ GATGCCGGAAAAACACCTTG 3′ and 5′ TGGCTGTACCCTTCCGCTT 3′. The primers for PP2Ac were 5′ CCA CAGCAAGTCACACATTGG 3′ and 5′ CAGAGCACTTGATCGCCTACAA 3′. The change in cycle threshold (C_T) value (ΔC_T) was derived by subtracting the C_T value for ribosomal protein L19 (RPL19), which served as an internal control, from the C_T values for PP2Ac. All reactions were run in duplicate by use of an ABI 7000 sequence detection system (Applied Biosystems). The difference in mRNA expression levels between HCV and non-HCV cells was expressed as an n-fold increase according to the formula $2\Delta^C T(non\text{-}HCV) - \Delta C_T(HCV)$.

Immunoprecipitation and immunoblotting. Cell lysates were incubated with anti-PP2Ac (Upstate), anti-PRMT1 (Abcam), anti-BIP (BD Biosciences, Basel, Switzerland), anti-NS4B (made by Darius Moradpour), or anti-GST (Amersham) antibodies overnight at 4°C. Protein A-Sepharose (Sigma) was added, and the samples were incubated for 3 h at 4°C on a rotating wheel. After sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transfer onto a nitrocellulose membrane (Schleicher & Schuell, Bottmingen, Switzerland), proteins were detected with anti-PP2Ac (Upstate) or anti-His antibodies (Santa Cruz). For loading amount control, the nitrocellulose membrane was stained using Blot-FastStain (GenoTech, Cell Concepts GmbH, Umkirch, Germany) according to the manufacturer's instructions.

GST-PRMT1 binding to PP2Ac. One ml of bacterial cell lysates expressing GST-PRMT1 was loaded onto a GST column (Amersham) and incubated for 10 min at room temperature. After centrifugation at 2,500 rpm for 1 min, 40 μg of total proteins from Huh7 cells was loaded onto the column to a final volume of 120 μl, and then the binding reaction was performed overnight at 4°C on a rotating wheel. The column was then washed four times with cold phosphate-buffered saline. For elution, 120 μl of reduced glutathione was used for 1 h at 4°C on a rotating wheel. To verify the specificity of the binding of PP2Ac to GST-PRMT1, the membrane was stripped and reblotted for BIP.

Methylation assay. An in vitro methylation assay was performed according to a protocol previously described by Mowen et al. in 2001 (30) with some modifications. For Fig. 1C, 50 μg of Huh7 lysate was incubated in the presence of 6 μg of GST-PRMT1 and 4 μl of ^{14}C-AdoMet in a final reaction volume of 80 μl for 2 h at 37°C. The reaction was stopped by adding 20 μl of sample loading buffer and boiling for 5 min. The proteins were separated on an 8% SDS-polyacrylamide gel. The lower part of the gel was cut out and stained with Coomassie blue to check for equal loading.

For methyltransferase activity measurements in UHCV-57.3 cells (Fig. 2C), the reaction was performed by using 10 μg of whole-cell lysate in the presence of 3 μl of ^{3}H-AdoMet for 2 h at 37°C. The reaction was then stopped by adding 20 μl of sample loading buffer, and the reaction volume was boiled for 5 min and separated on an 8% SDS-polyacrylamide gel. The upper part of the gel was fixed for 30 min in a solution of isopropanol:water:acetic acid (25:65:10) and then amplified in Amplify (Amersham) with gentle agitation for 30 min. The gel was dried and then exposed to X-ray Hyperfilm (Amersham) for 5 days. The lower part of the gel was cut out and stained with Coomassie blue to check for equal loading.

For in vitro methylation of purified NS3 helicase (Fig. 3), 30 μg of His-Hel-His (13) was incubated with 2 μg of GST-PRMT in the presence of 6 μl of ^{14}C-AdoMet for 2 h at 37°C. The reaction was then stopped by adding 20 μl of sample loading buffer, and the reaction volume was boiled for 5 min and separated on an 8% SDS-polyacrylamide gel.

DNA-DNA substrate. To prepare the double-stranded DNA (dsDNA) substrate for the unwinding assay, a short DNA oligonucleotide, 5′-TGG TAC TCC TCA CAC CTG GGC GGC GGT TAA-3′, was ^{32}P radiolabeled using the T4 polynucleotide kinase (Promega). Unincorporated ATP was removed through a Mini Quick Spin column. The labeled oligonucleotide was mixed with an equal amount the unlabeled complementary strand, 5′-GAC TAC GTA CTG TTA ACC GCC GCC CAG GTG TGA GGA GTA CCA GGC CAG ATC TGC-3′. The mixture was heated to 95°C for 3 min and left to cool slowly at room temperature.

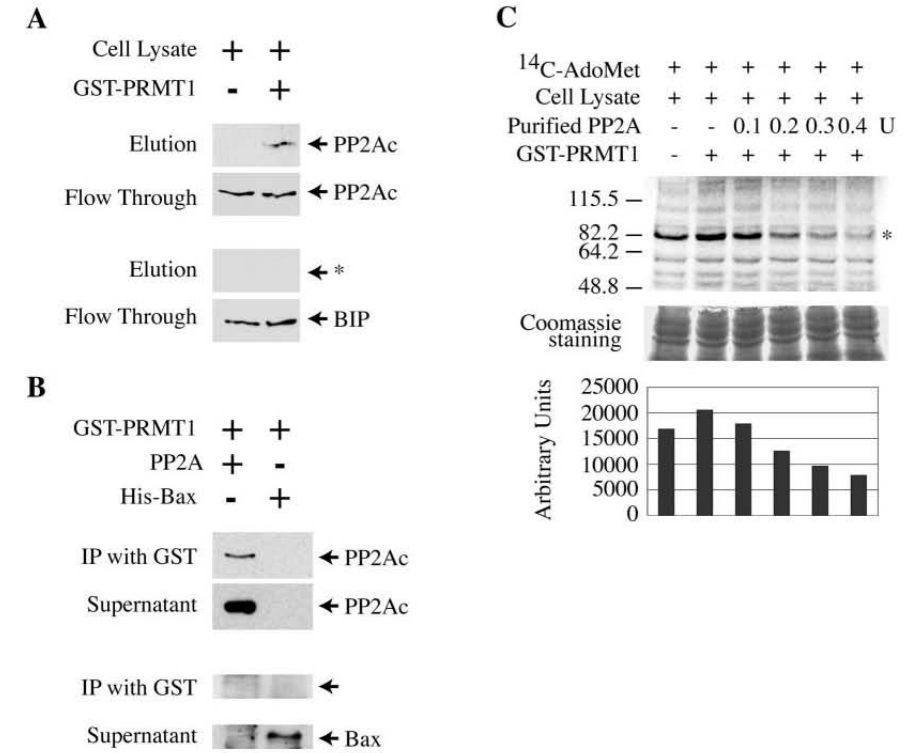

FIG. 1. PP2Ac binds directly to PRMT1 and inhibits its enzymatic activity. (A) Whole-cell lysate from Huh7 cells was incubated overnight at 4°C in a GST column that was left empty (left lane) or in one that was preloaded with GST-PRMT1 (right lane). The unbound proteins were recovered as flowthrough, and the bound proteins were eluted with reduced glutathione. PP2Ac was detected by Western blotting (upper panels). As a control, the membranes were then stripped and reprobed with antibodies against the immunoglobulin binding protein BIP (lower panels). The arrow with the asterisk shows the size of BIP. (B) Purified PP2Ac (0.2 units) or His-Bax (100 ng) was incubated with 10 μg of purified GST-PRMT1. Antibodies to GST were used to immunoprecipitate bound proteins. PP2Ac or His-Bax was detected by Western blotting (with anti-PP2Ac or anti-His, respectively). (C) Whole-cell lysate from Huh7 cells was incubated with ^{14}C-AdoMet alone (lane 1), with ^{14}C-AdoMet plus GST-PRMT1 (lane 2), or with ^{14}C-AdoMet plus GST-PRMT1 plus 0.1 units (lane 3), 0.2 units (lane 4), 0.3 units (lane 5), or 0.4 units (lane 6) of purified PP2Ac. The proteins were separated on an SDS-polyacrylamide gel, and methylation of proteins was detected by autoradiography. To control for equal loading, a 1-cm strip at the bottom of the gel was cut and stained with Coomassie blue (middle panel). The strongest band in the autoradiography (indicated with an asterisk) was quantified for each lane by use of NIH imaging software (lower panel).

DNA helicase assay. Purified methylated (Met-NS3h) or unmethylated (NS3h) His-Hel-His (100 nM) (13) was incubated with the double-stranded DNA substrate (10 nM) in reaction buffer (25 mM MOPS [morpholinepropanesulfonic acid; pH 6.2], 0.1% Tween 20, 3 mM $MgCl_2$) for 15 min at 23°C. The reactions were started by the addition of 5 mM ATP and then stopped at the indicated time points by the addition of a glycerol loading buffer containing 50 nM of a capture oligonucleotide (5′-TTA ACC GCC GCC CAG GTG TGA GGA GTA CCA-3′), 20 mM EDTA, and 0.5% SDS. The samples were analyzed by 8% nondenaturating polyacrylamide gel electrophoresis and quantitated by use of a PhosphorImager (Molecular Dynamics).

Purified methylated NS3h was obtained by incubating 30 μg of His-Hel-His (13) with 1 μg, 2 μg, or 3 μg of purified GST-PRMT in the presence of 9 mM AdoMet for 2 h at 37°C. To prepare the unmethylated NS3h, 30 μg of His-Hel-His was incubated with 2 μg of GST-PRMT1 for 2 h at 37°C (but without AdoMet).

ATPase assay. Reactions were done at 37°C in reaction buffer (25 mM MOPS [pH 6.2], 0.1% Tween 20, 3 mM $MgCl_2$, 5 mM ATP) with 100 nM of methylated or unmethylated NS3h. ATP quantification was done after 10 min with an ATP determination kit (Biaffin GmbH & Co KG, Kassel, Germany) according to the manufacturer's instructions.

Treatment of HCV replicon cell lines with the PP2A inhibitor okadaic acid. HuH-7.5 cells harboring a subgenomic HCV replicon (3, 4) (kindly provided by Charles M. Rice, The Rockefeller University, New York, NY) were treated for 18 h with 5 μl/ml dimethyl sulfoxide, 25 nM OA, 2 μM 2′-C-methyladenosine (6) (a specific inhibitor of the HCV RNA-dependent RNA polymerase; kindly provided by Steven S. Carroll, Merck & Co., Inc., West Point, PA), or 1,000-U/ml human IFN-α2a (Roferon-A; Roche). Preliminary dose titrations indicated that 25 nM OA strongly inhibited PP2A activity with only minimal toxicity after 18 h. Subsequently, total cellular RNA was extracted with Trizol (Invitrogen) following the manufacturer's instructions. Denaturing agarose gel electrophoresis and Northern blot analyses were performed according to standard protocols. ^{32}P-labeled cDNA fragments representing the entire HCV nonstructural region and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (12) as an internal control were employed simultaneously for Northern blot hybridization.

PP2Ac siRNA treatment of HuH-7.5 cells harboring a subgenomic HCV replicon. HuH-7.5 cells (10^6) were transfected using Lipofectamine 2000 from Invitrogen according to the manufacturer's instructions with 10 μl of a 20-pmol/μl solution of PP2Ac SMARTpool small interfering RNA (siRNA) duplexes from Dharmacon or with a corresponding amount of a nonsilencing siRNA duplex from QIAGEN. Four hours after the transfection, cells were recovered for 48 h in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Total RNA was isolated and reverse transcribed. RNA quantification was

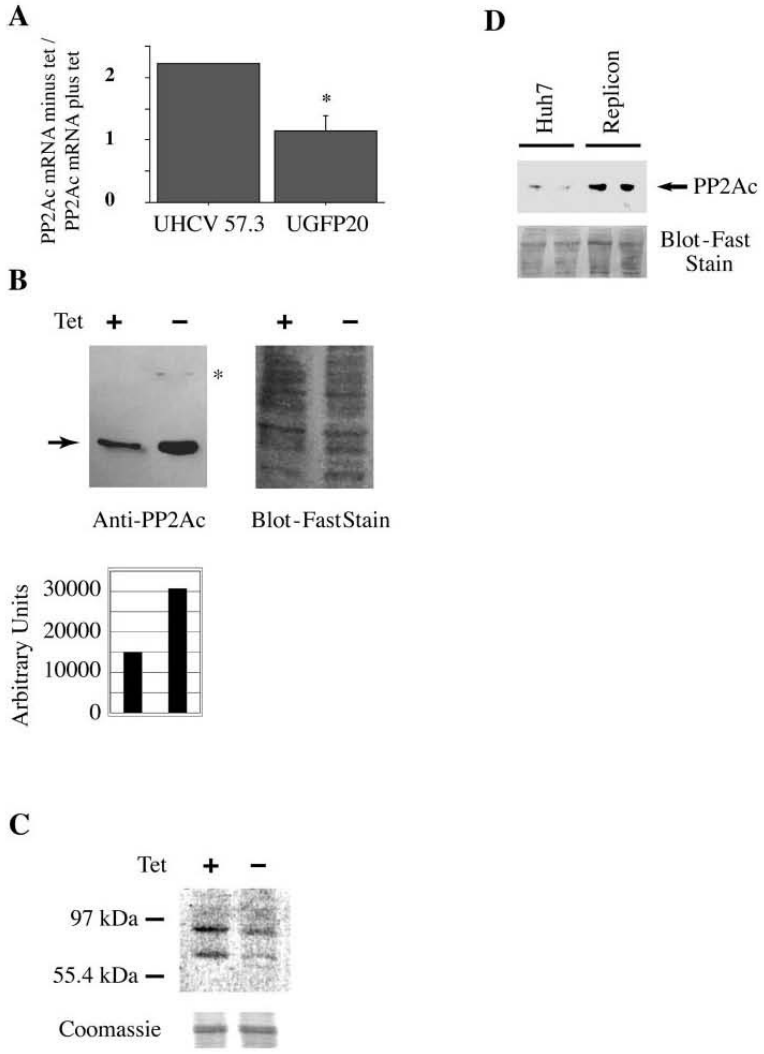

FIG. 2. Expression of HCV proteins induces PP2Ac. UHCV-57.3 and UGFP-20 cells (controls that express green fluorescent protein) were cultured for 24 h in the presence or absence of tetracycline. (A) PP2Ac mRNA was quantified with real-time RT-PCR. The ratio of the amounts of PP2Ac mRNA in derepressed (minus tet) and repressed cells (plus tet) is shown. Expression of viral proteins increases PP2Ac mRNA levels about twofold. The graph shows the mean (with the standard error of the mean) of two independent experiments. No error bar is shown for UHCV-57.3 cells, because the two values (2.22 and 2.21) were too close. (B) PP2Ac protein (arrow) expression in UHCV-32 cells was examined by Western blotting (left panel). Cells were cultured in presence (left lane) or absence (right lane) of tetracycline. Membranes were probed with a mixture of antibodies to PP2Ac (arrow) and NS4B (asterisk; faint upper band in right lane). The PP2Ac bands were quantified with NIH imaging software (lower panel). As a loading control, the membrane was stained with Blot-FastStain (right panel). (C) UHCV-57.3 cells were cultured in presence (left lane) or absence (right lane) of tetracycline for 24 h. Whole-cell lysates were then incubated for 2 h with radioactively labeled AdoMet. Protein methylation was detected by autoradiography. Equal loading was controlled by cutting the lower part of the gel and staining with Coomassie blue (lower panel). (D) Whole-cell extracts were prepared from two culture plates each of Huh7 cells and of Huh7 cells harboring an HCV replicon (Huh7.5 cells). The samples were analyzed by Western blotting using an antibody against PP2Ac (arrow). Loading was controlled by staining the membrane with Blot-FastStain (lower panel). Huh7.5 cells (lanes 3 and 4) had increased PP2Ac protein expression compared with Huh7 cells (lanes 1 and 2).

done by SYBR-PCR using specific primers for PP2Ac, HCV, and GAPDH. The primers for HCV were 5′ CGCTGCTTCTGCTTTCG 3′ and 5′ CACCCCTGC TCATAACC 3′. The primers for GAPDH were 5′ CAAGCTGTGGGCA AGGT 3′ and 5′ GGAAGGCCATGCCAGTGA 3′. The primers for PP2Ac and further details of the method are described above (see "RNA isolation, reverse transcription, and SYBR-PCR.")

PP2A phosphatase activity assay. Assessment of PP2A activity was performed according to the manufacturer's instructions with whole-cell extracts by use of a

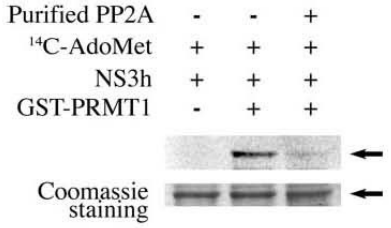

FIG. 3. PRMT1 methylates NS3 on its helicase domain, and PP2Ac inhibits the enzymatic activity of PRMT1. Ten μg of purified NS3 helicase domain was incubated with ^{14}C-AdoMet alone (lane 1), ^{14}C-AdoMet with 6 μg of GST-PRMT1 (lane 2), or ^{14}C-AdoMet with 6 μg of GST-PRMT1 plus 0.2 units of PP2Ac (lane 3).

serine/threonine phosphatase assay system (Promega) with PPTase-2A buffer containing 250 mM imidazole (pH 7.2), 1 mM EGTA, 0.1% β-mercaptoethanol, and 0.5 mg/ml bovine serum albumin.

RESULTS

PP2Ac binds directly to PRMT1 and inhibits its enzymatic activity. We have shown previously that expression of a constitutive active form of PP2Ac in Huh7 cells results in a strongly reduced methylation of STAT1 (10). Since STAT1 methylation is catalyzed by PRMT1 (30), we first wanted to test if PP2Ac can bind to PRMT1. To that end, whole-cell extracts from Huh7 cells were loaded onto a GST-PRMT1 column, and the flowthrough as well as the bound proteins were analyzed by Western blotting. As shown in Fig. 1A, PP2Ac was specifically bound by PRMT1 (whereas the ubiquitously expressed protein BIP was not). This interaction was direct and did not require additional proteins present in the whole-cell extract, because purified PP2Ac was also bound by GST-PRMT1 (Fig. 1B). As a control, purified His-Bax fusion protein was incubated with GST-PRMT1 but could not be coimmunoprecipitated (Fig. 1B). Next, we tested if PP2Ac binding to PRMT1 inhibits its methyltransferase catalytic activity. Whole-cell lysates from Huh7 cells were incubated with the radioactively labeled methyl group donor ^{14}C-AdoMet. In the in vitro methylation assay used, the transfer of ^{14}C-labeled methyl groups to cellular proteins by endogenous PRMT1 can be detected by autoradiography after separation of the proteins on an SDS-polyacrylamide gel (Fig. 1C, lane 1). The addition of purified GST-PRMT1 increases the methylation of proteins (Fig. 1C, lane 2). Importantly, the methylation of proteins in the lysate can be inhibited in a dose-dependent way by the addition of purified PP2Ac (Fig. 1C, lanes 3 to 6). We conclude that PP2Ac directly binds and inhibits PRMT1.

Expression of HCV proteins in cells up-regulates PP2Ac expression and inhibits cellular methyltransferases. We have shown previously an increased expression of the catalytic subunit of protein phosphatase 2A (PP2Ac) in liver extracts from HCV transgenic mice and in liver biopsy samples from patients with CHC (10). The significant increase of PP2Ac expression in these samples was surprising, since it has been reported that PP2Ac expression is very tightly regulated by an autoregulatory loop (1). To further study the effect of HCV proteins on PP2Ac expression, we used a well-characterized cell line that inducibly expresses the entire open reading frame of HCV (UHCV-57.3) (32). HCV protein expression in these cells can be repressed by adding tetracycline to the culture medium. In the absence of tetracycline, UHCV-57.3 cells express all HCV proteins (32). As shown in Fig. 2A, which depicts results of real-time quantitative reverse transcription-PCR (RT-PCR) analysis, HCV protein expression induced a twofold increase in the PP2Ac mRNA concentration. By contrast, no upregulation of PP2Ac was found in UGFP-20 cells, which inducibly express the green fluorescent protein as a nonrelevant control. This enhanced mRNA expression resulted in an increased PP2Ac protein expression level (Fig. 2B). Because PP2Ac expression levels are usually tightly controlled in cells, we speculated that the increase in PP2Ac expression induced by HCV protein expression is biologically relevant. We therefore measured the consequences of increased PP2Ac expression on methylation of cellular substrates using the methylation assay. Indeed, when cell extracts of repressed and derepressed UHCV-57.3 cells were incubated with ^{3}H-AdoMet, an overall reduction of endogenous methylase activity was found in HCV protein-expressing cells (Fig. 2C). Considering the in vitro assay results shown in Fig. 1C, we believe that the most likely explanation for this result is that HCV protein-induced overexpression of PP2Ac inhibits PRMT1. HCV-induced overexpression of PP2Ac was also found in HCV replicon cells (Fig. 2D).

PP2Ac inhibits NS3 methylation by PRMT1. Next, we tested whether GST-PRMT1 can methylate NS3. To this end, we expressed and purified a NS3 helicase protein (NS3h). Incubation of NS3h with purified GST-PRMT1 in the presence of ^{14}C-AdoMet led to methylation of NS3h (Fig. 3, lane 2). This result is in agreement with a previous report that NS3 is methylated on arginine 467 of the NS3 helicase domain (corresponding to position 1493 of the polyprotein) (31). Importantly, the addition of purified PP2Ac to the in vitro methylation assays inhibited the arginine methylation of the NS3 helicase domain (Fig. 3, lane 3).

Methylation of NS3 inhibits its helicase activity. To assess the effect of NS3 methylation on its helicase activity, we used methylated and unmethylated purified NS3 helicase (amino acids 166 to 631 of NS3) in a helicase assay (22). The substrate for the reaction was a radioactively labeled double-stranded DNA. Heating the dsDNA at 95°C for 5 min or unwinding the dsDNA by enzymatic activity of NS3 helicase produced a single-stranded DNA (Fig. 4). Methylated NS3 helicase was obtained by incubating purified NS3 helicase with 1 μg, 2 μg, or 3 μg of purified PRMT1 in the presence of the methyl donor AdoMet (9 mM) for 2 h at 37°C. To exclude an unspecific inhibition of NS3 helicase by PRMT1, the sample with the unmethylated NS3 helicase was also incubated with purified PRMT1 (2 μg) without the methyl donor AdoMet. When tested in the helicase assay, the unmethylated NS3 helicase was more active than the methylated samples (Fig. 4A, lane 2 versus lanes 3 to 5). The difference in the levels of unwinding activity was evident at all time points during a time course experiment (Fig. 4B). We then measured the levels of ATP consumption by the methylated and unmethylated NS3 helicases during a 10-min helicase assay experiment. The more active unmethylated NS3 helicase consumed 65% of the ATP, whereas the less active methylated NS3 helicase samples con-

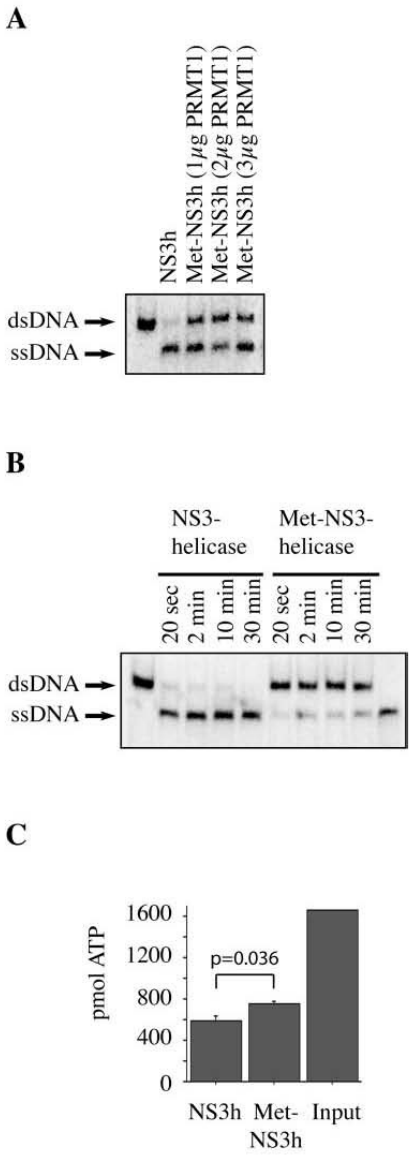

FIG. 4. Methylation of NS3 helicase inhibits its enzymatic activity. (A) Samples of 30 μg of purified NS3 helicase were incubated with 1 μg, 2 μg, or 3 μg of GST-PRMT1 and 9 nM AdoMet for 2 h at 37°C (lanes 3 to 5). The unmethylated NS3 helicase sample was also incubated with 2 μg of GST-PRMT1 for 2 h, but without AdoMet (lane 2). The samples were then used in a helicase assay with double-stranded DNA oligonucleotides as a substrate (lane 1). After 10 min, most of the dsDNA oligonucleotides were unwound to single-stranded DNA (ssDNA) by the unmethylated NS3 helicase (lane 2). In the cases of the methylated (Met-) NS3 helicase samples, about half the dsDNA substrates were still unwound after 10 min (lanes 3 to 5). (B) Time course experiment with unmethylated and methylated NS3 helicase. The enzymes were incubated with dsDNA substrate for the times indicated in the figure. In the samples with methylated NS3 helicase, a large proportion of the dsDNA substrate remains unwound (lanes 5 to 9). In the first and the last lanes, the substrate dsDNA and single-stranded DNA obtained by heating dsDNA, respectively, are shown. (C) ATP hydrolysis assay. Methylated or unmethylated NS3 helicase (100 nM) was incubated with 5 mM ATP. The amount of the unconsumed ATP was measured after 10 min. The unmethylated enzyme (lane 1) consumed 65%, and the methylated enzyme (lane 2) consumed about 55% of the input ATP.

sumed only 55% of the ATP. The difference was statistically significant (Fig. 4C).

We conclude that methylation of NS3 by PRMT1 inhibits its helicase activity. Theoretically, this conclusion could be tested more vigorously by doing in vitro helicase assays with a mutated NS3 that cannot be methylated on arginine 467 and therefore should not be inhibited by preincubation with PRMT1. However, the mutation of arginine 467 in the NS3 helicase domain leads to a complete loss of helicase activity (16). Because PP2Ac can inhibit PRMT1, as shown above, the upregulation of PP2Ac by HCV ultimately may result in enhanced helicase activity of NS3.

If PP2A enhances NS3 helicase activity, then inhibition of PP2A should have consequences for HCV replication. We therefore used HuH-7.5 cells harboring a subgenomic HCV replicon (3, 4) and the PP2A inhibitor OA to test this hypothesis. OA was added to the cells at a final concentration of 25 nM. This concentration was used because it significantly inhibited the phosphatase activity of PP2A with only minimal toxicity (data not shown). Treatment of replicon cells with OA indeed inhibited the replication of the HCV replicon (Fig. 5, lane 3). This inhibition was not as strong as that seen after treatment with IFN-α or with the HCV polymerase inhibitor 2′-*C*-methyladenosine (Fig. 5, lanes 4 and 5) but was still highly significant.

To confirm these results, PP2Ac expression was inhibited in HuH-7.5 cells by use of siRNA (Fig. 6). Again, a reduction of replicon RNA to 70% of the initial value found for HuH-7.5 cells was observed.

DISCUSSION

Given the limited coding capacity of a 9.6-kb RNA genome, HCV has an astonishing ability to evade the powerful immune system of the host and to establish a persistent infection over decades. In its interference with host defense mechanisms, HCV therefore probably targets a limited number of key enzymes of the cells in which it replicates. Here we provide evidence that PP2A is such a key enzyme. We have found that HCV infection or expression of HCV proteins induces the expression of PP2Ac in liver biopsy samples from patients with chronic hepatitis C (10), in liver cells of HCV transgenic mice (10), and in UHCV-57.3 and UHCV-32 cells. PP2A is a widely expressed serine/threonine phosphatase involved in a wide range of cellular processes (15, 27, 34). It can be expected that the induction of such an important phosphatase will have a number of effects on the host cell. Here we have concentrated our efforts on the study of the effect of PP2A on PRMT1, an important arginine methyltransferase. During previous studies, we found that HCV interferes with IFN-α-induced signaling by inhibiting the methylation of STAT1, an important signal

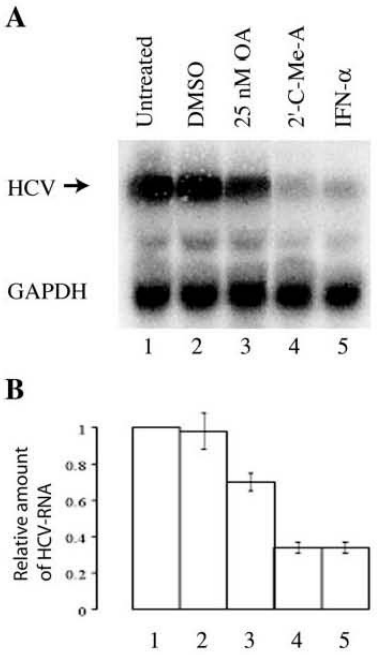

FIG. 5. HuH-7.5 cells harboring a subgenomic HCV replicon were left untreated or were treated for 18 h with 5 μl/ml dimethyl sulfoxide (DMSO), 25 nM OA, 2 μM 2′-*C*-methyladenosine (2′C-Me-A), or 1,000 U/ml human IFN-α2a as indicated. Eight μg of total cellular RNA per lane was analyzed by 1.2% denaturing agarose gel electrophoresis, which was followed by Northern blotting using HCV- and GAPDH-specific probes. (A) Representative Northern blot. (B) The Northern blot shown in panel A and two additional blots from a representative experiment performed in triplicate were quantified by phosphorimaging. The mean intensity (± standard deviation) of each HCV-specific band relative to that of each GAPDH-specific band is shown.

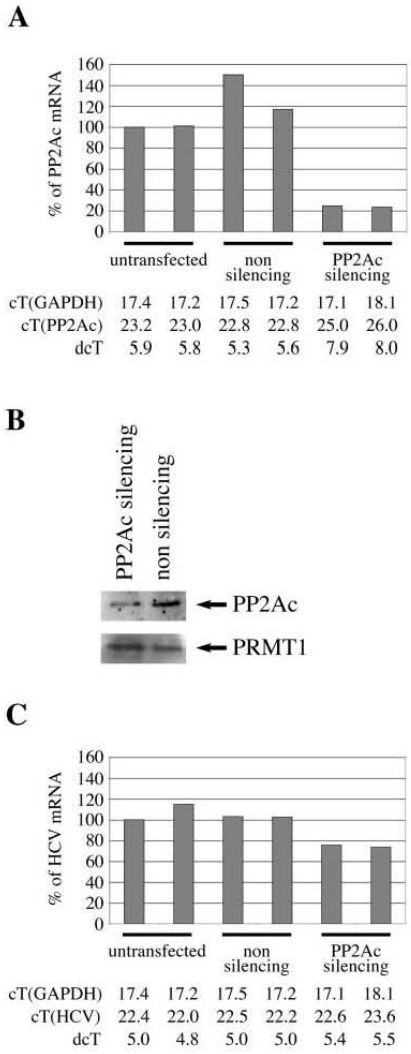

FIG. 6. Suppression of PP2Ac expression by siRNA treatment of HuH-7.5 cells inhibits the replication of the HCV replicon. HuH-7.5 cells (two plates each) were left untransfected or were transfected with a nonsilencing or PP2Ac-silencing oligonucleotide, as indicated. (A) The expression of PP2Ac mRNA was quantified with real-time RT-PCR. (B) The expressions of PP2Ac and of PRMT1 proteins were analyzed by Western blotting. (C) The expression of HCV replicon RNA was quantified with real-time RT-PCR. In panels A and C, the expression levels of PP2Ac and HCV replicon found in one of the untransfected cells was set to 100% (reference sample), and the expression levels found in the other five samples are shown relative to that of the reference sample. Below panels A and C are the measured C_T and ΔC_T (dcT) values.

transducer of IFN-α. Since STAT1 methylation is catalyzed by PRMT1, we hypothesized that PP2A directly or indirectly inhibits the enzymatic activity of PRMT1. We show here with purified proteins that PP2Ac binds directly to PRMT1 and, furthermore, that PP2Ac binding inhibits the methyltransferase activity of PRMT1 in vitro. Therefore, as a first and important consequence of PP2Ac induction by HCV, STAT1 methylation is reduced. Unmethylated STAT1 is bound by its inhibitor PIAS1, and IFN-α-induced induction of target genes important for cellular defense against HCV is impaired.

Surprisingly, the same mechanism of interference with the host cell is exploited twice by HCV. It has been shown recently that NS3, an HCV protein with protease and helicase activity, can be modified by arginine methylation through the cellular enzyme PRMT1 (31). The functional relevance of this modification was not known, but mutational analysis of arginine residues in the helicase domain provided strong evidence that these arginines are important for the enzymatic activity (16). For example, the mutation of arginine residue 467 of NS3 helicase (arginine 1493 in the polyprotein) to lysine disrupts the enzymatic activity completely (16). Here we show that methylation of NS3 reduces its helicase activity but does not completely abrogate it. Given the direct interaction of PP2Ac with PRMT1, it was not surprising that we did find an inhibition of NS3 methylation by PP2Ac. We conclude that, as a

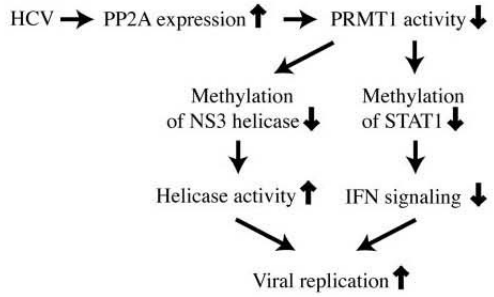

FIG. 7. Enhanced HCV replication is a consequence of both the increase of NS3 helicase activity and the inhibition of IFN-α-induced Jak-STAT signaling.

second important consequence of PP2Ac induction by HCV, NS3 helicase activity is increased.

The characterization of PP2A as a key target of HCV interference with the host cell identifies PP2A as a potential target of treatment strategies against the virus. In a proof-of-concept experiment, we treated replicon cells with OA, a reasonably specific inhibitor of PP2A (27), and found a significant inhibition of replication. The same degree of inhibition of the replicon was found after decreasing the expression of PP2Ac by siRNA. In our model (Fig. 7), inhibition of PP2A by OA results in an enhanced enzymatic activity of PRMT1. NS3 helicase is one of the substrates of PRMT1 and becomes methylated on critical arginine residues. This methylation reduces the helicase activity of NS3, leading to a decrease of viral (or replicon) replication. There are two caveats that we would like to mention. First, compared to IFN-α or the HCV polymerase inhibitor, OA is a relatively weak inhibitor of replication. After 18 h of OA treatment, a 30% reduction of the amount of replicon RNA in the cell was observed. This is clearly less than the 70% inhibition observed after treatment with IFN-α or the HCV polymerase inhibitor. However, in the long course of a natural HCV infection, even relatively small increases or decreases of viral replication activity can make a big difference on the virus-host interaction. Second, we would also like to stress that, given the numerous other functions of PP2A in cell homeostasis, we cannot exclude that inhibition of the HCV replicon by OA or by siRNA inhibition of PP2Ac expression involves mechanisms other than those described here. However, our in vitro data obtained with purified proteins show that these proteins can and do interact, and it should be worthwhile to further study interventions targeted to disrupt some of the HCV-induced changes of PP2Ac expression or of PRMT1 activity.

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3.2 S-adenosylmethionine and Betaine Correct Hepatitis C Virus Induced Inhibition of Interferon Signaling *In Vitro*

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S-Adenosylmethionine and Betaine Correct Hepatitis C Virus Induced Inhibition of Interferon Signaling *In Vitro*

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Hepatitis C virus (HCV) infection is an important cause of chronic liver disease. Standard therapy, pegylated interferon α (pegIFNα) combined with ribavirin, results in a sustained response rate in approximately half of patients. The cause of treatment failure in the other half of the patients is unknown, but viral interference with IFNα signal transduction through the Jak-STAT pathway might be an important factor. We have shown previously that the expression of HCV proteins leads to an impairment of Jak-STAT signaling because of an inhibition of STAT1 methylation. Unmethylated STAT1 is less active because it can be bound and inactivated by its inhibitor, protein inhibitor of activated STAT1 (PIAS1). We show that treating cells with S-adenosyl-L-methionine (AdoMet) and betaine could restore STAT1 methylation and improve IFNα signaling. Furthermore, the antiviral effect of IFNα in cell culture could be significantly enhanced by the addition of AdoMet and betaine. In conclusion, we propose that the addition of these drugs to the standard therapy of patients with chronic hepatitis C could overcome treatment resistance. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006;43:796-806.)

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide.[1] Chronic hepatitis C (CHC) may lead to cirrhosis and hepatocellular carcinoma. Type I interferons (IFNs) are crucial and potent components of the early host response against virus infection[2] and recombinant pegylated IFNα2a and IFNα2b are widely used for the treatment of CHC and chronic hepatitis B. Current standard treatment with pegylated IFNα and ribavirin can cure about 50% of patients with CHC.[3,4] The cause of treatment failures in half of the patients is not fully understood, but viral interference with IFNα signal transduction from the cell surface to the nucleus may be an important factor. The most important signal transduction pathway for IFNα is the Jak-STAT pathway (Fig. 1A).[5] Signal transducers and activators of transcription (STAT) proteins are activated by members of the Jak kinase family through the phosphorylation of a single tyrosine residue.[6] Activated STATs form dimers, translocate into the nucleus and bind specific DNA elements in the promoters of target genes.[7,8] STATs are deactivated by tyrosine dephosphorylation in the nucleus, followed by the decay of dimers and the nuclear export of STATs.[9,10] Important negative regulators of this signal transduction pathway have been found at two levels. First, the suppressor of cytokine signaling (SOCS) family members, SOCS1 and SOCS3, prevent phosphorylation and activation of IFNα induced STATs by inhibiting the IFNα receptor associated Jak kinases.[11] Second, downstream of STAT activation by tyrosine phosphorylation, IFNα induced gene transcription can be inhibited by protein inhibitor of activated STAT1 (PIAS1). PIAS1 inhibits binding of STAT1 dimers to the response elements in the promoters of target genes (Fig. 1B).[12,13] The binding of PIAS1 to STAT1 is regulated by methylation of STAT1 by protein arginine methyl transferase 1 (PRMT1).[14] Arginine methylation inhibits binding of PIAS1 to STAT1, whereas demethylation of STAT1 enhances its association with PIAS1.

Abbreviations: HCV, hepatitis C virus; CHC, chronic hepatitis C; IFN, interferon; STAT, signal transducers and activators of transcription; SOCS, suppressor of cytokine signaling; PIAS1, protein inhibitor of activated STAT1; PRMT1, protein arginine methyltransferase 1; PP2Ac, catalytic subunit of protein phosphatase 2a; AdoMet, S-adenosyl-L-methionine; AdOx, adenosine dialdehyde; CT, threshold cycle; GST, glutathione S-transferase; AdoHcy, S-adenosyl-L-homocysteine; ISG, IFN stimulated genes; VSV, vesicular stomatitis virus; GSH, glutathione.

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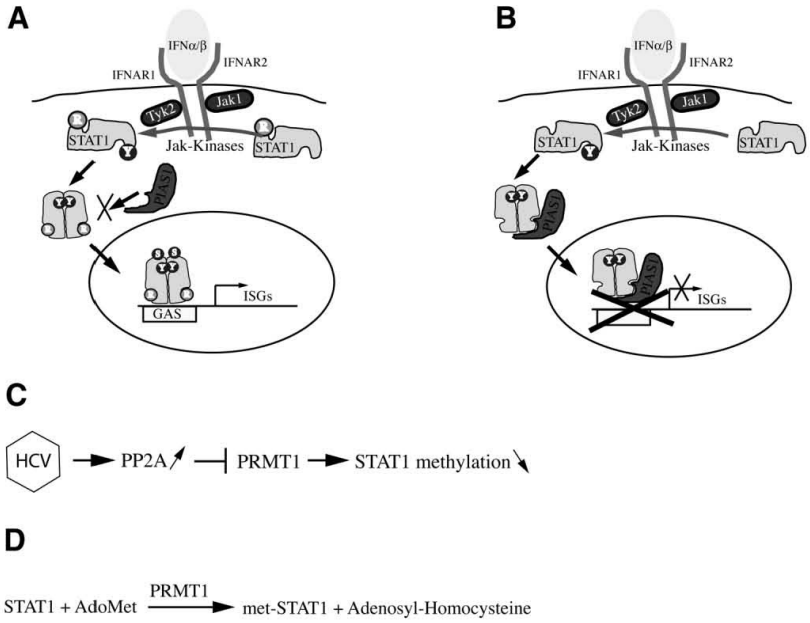

Fig. 1. IFNα signaling. Binding of IFNα to its receptor on the cell surface induces the activation of the phosphotyrosine kinases Jak1 and Tyk2. STAT1 is recruited to the receptor kinase complex and activated by phosphorylation on tyrosine 701. STAT1 then forms dimers, translocates into the nucleus, and binds to promoter elements of IFNα stimulated genes (ISGs).The protein inhibitor of activated STAT1 (PIAS1) can not bind to STAT1 dimers because the STATs are methylated on an arginine. (A) Unmethylated STAT1 molecules can also be recruited to the receptor kinase complex, and are phosphorylated normally. However, PIAS1 can bind to STAT1 dimers and lower their binding affinity especially for weak STAT1 binding sites in ISG promoters. A number of ISGs are not upregulated when STAT1 is bound by PIAS1.[1]. (B) Working model of HCV interference with IFNα signaling. HCV infection induces the overexpression of protein phosphatase 2A catalytic subunit (PP2Ac). PP2Ac inhibits protein arginine methyltransferase 1 (PRMT1). As a consquence, STAT1 methylation is reduced. (C) Adenosylmethionine is the methyl group donor for STAT1 methylation catalyzed by PRMT1.

We have shown previously that the expression of HCV proteins in human osteosarcoma cell lines or in liver cells of transgenic mice inhibits IFNα induced signaling through the Jak-STAT pathway.[15,16] HCV induced a hypomethylation of STAT1 that resulted in an increased binding of STAT1 to PIAS1.[17] HCV proteins also induced the expression of the catalytic subunit of protein phosphatase 2A (PP2Ac), and PP2Ac overexpression was found to be sufficient to induce STAT1 hypomethylation.[17] Figure 1C shows our current model of HCV interference with IFNα signaling. Based on these results, we hypothesized that pharmacological treatment that increases the methylation of STAT1 could improve IFN signaling. Interestingly, the methyl group donor for STAT1 methylation by PRMT1 is S-adenosy-L-methionine (AdoMet or SAMe) (Fig. 1D), a compound that has been used to treat alcoholic liver disease and is available in many countries as a nonprescription drug.

To explore the potential of AdoMet for improving the efficacy of peg IFNα in the treatment of patients with CHC, we wanted to test if (1) PP2Ac is overexpressed in liver biopsies of patients with chronic hepatitis, (2) PP2Ac inhibits PRMT1 directly, (3) STAT1 methylation can be induced by enhancing the intracellular concentration of AdoMet, and (4) such a treatment improves IFNα–induced signaling and increases the antiviral effect of IFNα in cell culture.

Materials and Methods

Reagents, Antibodies, Cells, and Plasmids. Human IFNα (hIFN; Roferon) was obtained from Hoffmann LaRoche (Basel, Switzerland). Purified PP2A and purified histone H4 were purchased from Upstate (LucernaChem, Lucerne, Switzerland). Purified Bcl2 was obtained from SantaCruz (LabForce AG, Nunningen, Switzerland). S-Adenosyl-L-Methionine (AdoMet), betaine and adenosine dialdehyde (AdOx) were from Sigma (Fluka Chemie GmbH, Buchs, Switzerland). ^{14}C-AdoMet was obtained from Amersham Biosciences (Amersham Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland). PIAS1 antibodies were a gift from Dr. K. Shuai. pGEX-HRMT1L2 (human PRMT1) expression vector was a gift from Dr. P. A. Silver. UHCV57.3 cells were from Dr. D.

Moradpour. UHCV57.3 cells are tetracycline-regulated cell lines containing the entire HCV open reading frame derived from a HCV H consensus cDNA.[18] Huh7 harboring the HCV replicon I_{377}/NS3-3′(clone 9-13) were from Dr. R. Bartenschlager.[19] HA-PP2Ac cells were previously described.[17]

Patients and Biopsies. From August, 2002, to April, 2005, all patients with CHC referred to the outpatient liver clinic of the University Hospital Basel and who had a liver biopsy were asked for their permission to use part of the biopsy for this study. The protocol was approved by the ethical commission of Basel. Written informed consent was obtained from all patients that agreed to participate in the study. Sample handling and extraction procedures have been previously described.[17] Control samples were from patients who underwent ultrasound-guided liver biopsies of focal lesions (mostly metastases of carcinomas) and who were asked for their permission to obtain a biopsy from the normal liver tissue outside the focal lesion. Only samples with histologically confirmed absence of liver disease were used as controls. The relevant clinical and histological data of the HCV patients are shown in Supplementary Table 1. (Supplementary material can be found on the HEPATOLOGY website: http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html.)

Expression and Purification GST-PRMT1. Bacterial cells were grown overnight in Luria-Bertani medium supplemented with 100 μg/mL ampicillin until OD 0.6-0.7. Glutathione S-transferase (GST)-PRMT1 expression was induced with 100 mmol/L IPTG for 3 hours at 37°C. After cell lysis the protein was purified using GST microSpin column (Amersham) according to manufacturer's instructions.

Preparation of Extracts From Cells and Liver Biopsies. Whole cell lysates and nuclear extracts were done as described.[17] The liver biopsies were homogenized in 100 μL of lysis buffer (100 mmol/L NaCl, 50 mM Tris pH 7.5, 1 mmol/L EDTA, 0.1 % Triton X-100, 10 mmol/L NaF, 1 mmol/L PMSF, and 1 mmol/L sodium orthovanadate), and the lysates were then centrifuged at 14,000 rpm for 5 minutes. Protein concentrations were determined with the BioRad Protein Assay (Bio-Rad Laboratories AG, Reinach, Switzerland).

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting was done as described.[17] To measure PP2Ac expression in human liver biopsies, 20, 50, and 100 ng of purified PP2Ac was loaded on each gel. These 3 samples allowed the calculation of a standard curve for each gel (Fig. 1A). The intensity of each band was analyzed by densitometry analysis using NIH Image software. The amount of PP2Ac in each liver biopsy was then determined according to the standard curve.

Electrophoretic Mobility Shift Assays. 1 μg nuclear extracts aliquots were used for EMSAs. SIE-m67 was used as the oligonucleotide probe.[15] STAT1 was supershifted with antibody SC346 from Santa Cruz (LabForce AG, Nunningen, Switzerland).

RNA Isolation, Reverse Transcription, and SYBR-PCR. Total RNA was isolated from the cells using Perfect RNA Eukaryotic Mini Kit (Eppendorf, Vaudaux-Eppendorf, Basel, Switzerland) according to manufacturer's instructions. RNA was reverse transcribed by M-MLV reverse transcriptase (Promega, Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Promega) and dNTPs. The reaction mixture was incubated for 5 minutes at 70°C and then for 1 hour at 37°C. The reaction was then stopped by heating at 95°C for 5 minutes. SYBR-PCR was performed based on SYBR-Green Fluorescence (SYBR-Green PCR Master Mix, Applied Biosystems, Foster City, CA). To prevent influence from genomic DNA amplification, the primers were designed across exon-intron junctions. The primers for GAPDH were 5′-GCTCCTCCTGTTCGACAGTCA-3′ and 5′-ACCTTCCCCATGGTGTCTGA-3′. The primers for tubulin were 5′-GCCAGTGCGGGAACCA-3′ and 5′-GGTCGATGCCGTGCTCAT-3′. The primers for IP10 were 5′-CGATTCTGATTTGCTGCCTTATC-3′ and 5′-GCAGGTACAGCGTACGGTTCT-3′. The primers for HCV were 5′-CACCCCTGCTCCATAACC-3′ and 5′-CGCTGCTTCTGCTTTCG-3′. The ΔCT value was derived by subtracting the threshold cycle (CT) value for GAPDH or tubulin, which served as an internal control, from the CT values for HCV or IP10, respectively. All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems). mRNA expression level of IP10 or HCV was expressed as a fold increase or fold decrease according to the formula $2^{\Delta CT(PBS)-\delta CT(Treatment)}$.

Methylation Assay. In vitro methylation assay was performed according to a protocol previously described[14] with some modifications. Briefly, 4 μg of purified histone H4 was incubated in the presence or in the absence of 5 μg of GST-PRMT1 and 10 μL of ^{14}C-AdoMet in a final reaction volume of 50 μg for 2 hours at 37°C. For the inhibition of GST-PRMT1 activity, 14 μg of purified PP2A was incubated with GST-PRMT1 for 40 minutes at 37°C prior to add to H4 and ^{14}C-AdoMet. Fourteen micrograms of purified Bcl2 was used as a negative control. The reaction was then stopped by adding 20 μL of a sample loading buffer, boiled for 5 minutes, and then separated on a 8 % SDS-polyacrylamide gel. The gel was dried and then exposed to PhosphorImager (Kodak) for 2 days.

Vesicular Stomatitis Virus Infection and Cell Viability. UHCV57.3 cells (30,000 cells) were cultured in 96 well plates for 24 hours in the absence of tetracycline to induce the expression of HCV proteins. During the last 3 hours, AdoMet (170 nmol/L) and betaine (50 μmol/L) were added. They were then infected with 10 pfu of vesicular stomatitis virus (VSV). From the time point of the infection cells were immediately treated with hIFNα (1000 U/mL) alone or in combination with 170 nmol/L AdoMet and 50 μmol/L betaine for 24 hours. 15 μL of the supernatant were then used to infect Vero cells (30,000 cells, 96 well plates) for 24 hours. Vero cell viability was determined using CellTiter 96 Aqueous nonradioactive cell proliferation assay (Promega) according to manufacturer's instructions. All samples were done in duplicate.

Plaque Assay. UHCV57.3 cells were cultured in 150 mm plate for 7 hours in the absence of tetracycline to induce the expression of HCV proteins (Supplementary Fig. 2). Cells were trypsinized and seeded in 24 well plates (360,000 cells/well) in the absence of tetracycline for another 17 hours before pretreated with 170 nmol/L AdoMet and 50 μmol/L Betaine for 3 hours. They were then infected with 1 pfu of VSV. From the time point of infection, cells were treated with hIFNα (10, 50, 100, or 1,000U/mL) alone or in combination with 170 nmol/L AdoMet and 50 μmol/L Betaine for 24 hours. Supernatants were collected and used (1:200) to infect Vero cells (monolayer, 6 well plates). At 1 hour post infection, supernatant was removed and 3% methylcellulose was overlayed. At 20 hours post infection, overlay was removed, cells were fixed with 4% formaldehyde for 30 minutes, and stained with 0.2% crystal violet in 20% methanol. Plaques were counted and multiplied by the dilution factor to determine viral titer as pfu/mL.

Results

Expression of PP2Ac in Human Liver Biopsies From Patients With CHC. Our working model (Fig. 1) of HCV interference with IFNα signaling was mainly based on studies with cultured cells and transgenic mice. We therefore aimed to investigate the relevance of this model for patients with CHC. We have previously published preliminary data showing that the expression level of PP2Ac is higher in liver biopsy samples from patients with CHC compared with controls.[17] To confirm this observation, we developed a semi-quantitative Western blot method (Fig. 2A-B) and measured PP2Ac expression in a large number of new biopsy samples (Fig. 2C). The median concentration of PP2Ac was 6.5 ng/μg total protein in 96 samples from patients with CHC, and 4.5 ng/μg total protein in 25 control samples (Fig. 2C). The difference was statistically significant with a P value of .0033 (Mann-Whitney U test). Of note, the expression

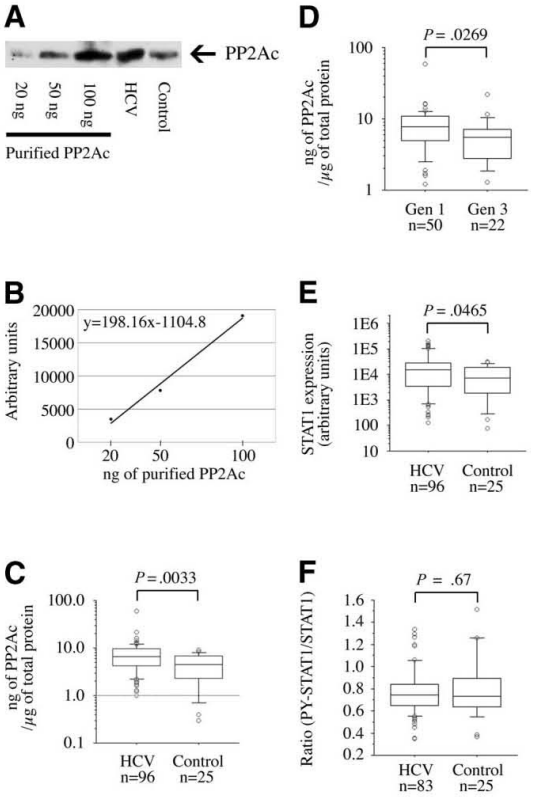

Fig. 2. Expression of protein phosphatase 2A catalytic subunit (PP2Ac) and STAT1 in human liver biopsies of patients with chronic hepatitis C. (A) Representative example of a Western blot showing the samples with 20ng, 50ng and 100ng of purified PP2Ac used to generate a calibration curve and one sample of a patient with chronic hepatitis C and a control sample from a patient without parenchymal liver disease. (B) Example of a calibration curve. The Western blot signal intensities were measured using NIH Image Software. (C) Box-plot diagram of quantified PP2Ac expression levels in liver biopsies of 96 patients with chronic hepatitis C and from 25 control patients without parenchymal liver disease. The difference in the expression of PP2Ac between HCV and control groups is statistically significant (P= .0033, Mann-Whitney U test). (D) PP2Ac expression is higher in samples from patients infected HCV genotype 1 compared with genotype 3. The difference is statistically significant (PI =.0269, Mann-Whitney U test). (E) Semiquantitative assessment of STAT1 expression. The STAT1 signal intensity in Western Blots was quantified with NIH Image Software. The results (arbitrary units) are shown in a box plot diagram. The difference between controls and HCV patients is statistically significant (P = .0465, Mann-Whitney U test). (F) Semiquantitative assessment of STAT1 tyrosine phosphorylation. The PY-STAT1 signal intensity (arbitrary units) and STAT1 expression levels (arbitrary units) in Western Blots was quantified with NIH Image Software. For each liver biopsy sample, the ratio of phosphorylated STAT1 to total STAT1 was calculated. The results are shown in a box plot diagram. The difference between controls and HCV patients is not statistically significant (P = .67, Mann-Whitney U test).

levels of PP2Ac in CHC samples varied considerably. We analyzed if age, sex, viral load, histological grade of inflammation, or stage of fibrosis were correlated with the expression levels of PP2Ac, but could not find any statistically significant correlation (Supplementary Table 1 and Supplementary Fig. 1). Only the presence or absence of HCV infection and the HCV genotype were significantly correlated with PP2Ac expression.

An increased expression level of PP2Ac is important in the context of CHC because PP2Ac over-expression in cultured cells inhibits IFNα signaling (Fig. 1).[17] Based on these observations, we propose that patients with a low or normal PP2Ac expression level would respond better to IFNα-based therapies than patients with high PP2Ac expression levels. An ongoing study will directly test this hypothesis. Indirect evidence for this hypothesis can be obtained by comparing PP2Ac expression levels in genotype 1 versus genotype 3 infected patients. Clinical studies have consistently shown that the patients infected with genotype 1 have a lower sustained response rate to treatments with (pegylated) IFNα plus ribavirin than patients infected with genotype 2 or 3.[3,4,20] In our group of patients, we found significantly higher expression levels of PP2Ac in 50 samples from patients infected with HCV genotype 1 (median = 7.8 ng PP2Ac/μg total protein) compared with 22 samples from patients infected with genotype 3 (median = 5.5 ng PP2Ac/μg total protein; $P = .027$), further supporting an important role of PP2A in HCV induced IFNα resistance (Fig. 2D).

We also determined the expression level of STAT1 in 96 biopsies from HCV patients and the phosphorylation of STAT1 in 83 HCV samples and 25 controls. STAT1 expression was increased in HCV samples (Fig 2E), and the phosphorylation of STAT1 was intact (Fig. 2F).

PP2Ac Directly Binds to and Inhibits PRMT1. These results are consistent with our previous work where we showed the block in IFNα signaling is not caused by a degradation of STAT1 or by an inhibition of STAT1 phosphorylation, but is further downstream in the signaling pathway, at the level of STAT1-PIAS1 association (Fig. 1). The reversible association of STAT1 with PIAS1 is regulated by STAT1 methylation.[14] We have previously shown that HCV protein expression inhibited the methylation of STAT1 and thereby increased the binding of PIAS1 to STAT1.[17] Because STAT1 methylation is catalyzed by PRMT1, we wanted to test if PP2Ac could directly inhibit the enzymatic activity of PRMT1. To this end, we expressed and purified PRMT1 and PP2Ac and used the purified proteins in an *in vitro* methylation assay. As shown in Fig. 3A, the methylation of histone H4 (a standard substrate for this assay) by PRMT1 is completely inhibited by PP2Ac. Based on these *in vitro* data we conclude that the upregulation of PP2Ac by HCV leads to an inhibition of PRMT1 (Fig. 1C). As a consequence, the methylation of many cellular proteins including STAT1 is reduced.

AdoMet and Betaine Increase Methylation of STAT1. AdoMet is available in many countries as an over-the-counter drug used for the treatment of liver diseases, especially alcoholic and nonalcoholic steatohepatitis. It is used as a precursor for glutathione biosynthesis. AdoMet depletion has been observed in a number of experimental models of liver diseases associated with increased oxidative stress and a depletion of reduced glutathione (GSH).[21-24] Oral AdoMet treatment has therefore been used in animal models and in patients to restore GSH content in the liver with the aim to treat liver diseases.[25-27] We were interested in AdoMet not because of its role in GSH biosynthesis,[28,29] but because PRMT1 uses AdoMet as the methyl group donor for STAT1 methylation (Fig. 1D). We hypothesized that AdoMet could correct the HCV induced STAT1 hypomethylation by shifting the STAT1 + AdoMet $\rightleftharpoons$ Met-STAT1 + S-adenosyl-L-homocysteine reaction equilibrium to the right. To test this hypothesis, we treated HA-PP2Ac cells with AdoMet. HA-PP2Ac cells are stably transfected with a constitutive active catalytic subunit of PP2A, HA-PP2Ac.[17] We have shown previously that IFNα induced signaling through the Jak-STAT pathway is inhibited in these cells because of hypomethylation of STAT1 and increased binding of PIAS1 to STAT1.[17] Here we show AdoMet could correct the inhibition of IFNα signaling in HA-PP2Ac cells (Fig. 3B). The methylation of STAT1 could be restored to levels found in naïve Huh7 cells, and the increased association of STAT1 with PIAS1 observed in HA-PP2Ac cells was returned to normal levels after treatment with AdoMet (Fig. 3B).

The intracellular AdoMet concentration can also be raised by treating cells with betaine.[30-32] Betaine (trimethyl-glycine) is the principle methyl donor for the generation of methionine from homocysteine, a reaction that is central to the recycling of AdoMet (Fig. 4).[33] In fact, treatment of HA-PP2Ac cells with betaine could restore STAT1 signaling just as well as AdoMet (Fig. 3C).

We then tested the effect of AdoMet and betaine on IFNα signaling in UHCV57.3 cells. UHCV57.3 cells inducibly express all HCV proteins.[34] In these cells, IFNα signaling is impaired by viral protein expression (Fig. 3D, lane 4).[15] However, pretreatment of cells with AdoMet and/or betaine restores STAT1 DNA binding even in the presence of viral proteins (Fig. 3D, lanes 5 to 7). To test if the increased DNA binding also improves the transcriptional activation of ISGs, we measured IP-10 mRNA levels after stimulation of cells with IFNα with or without

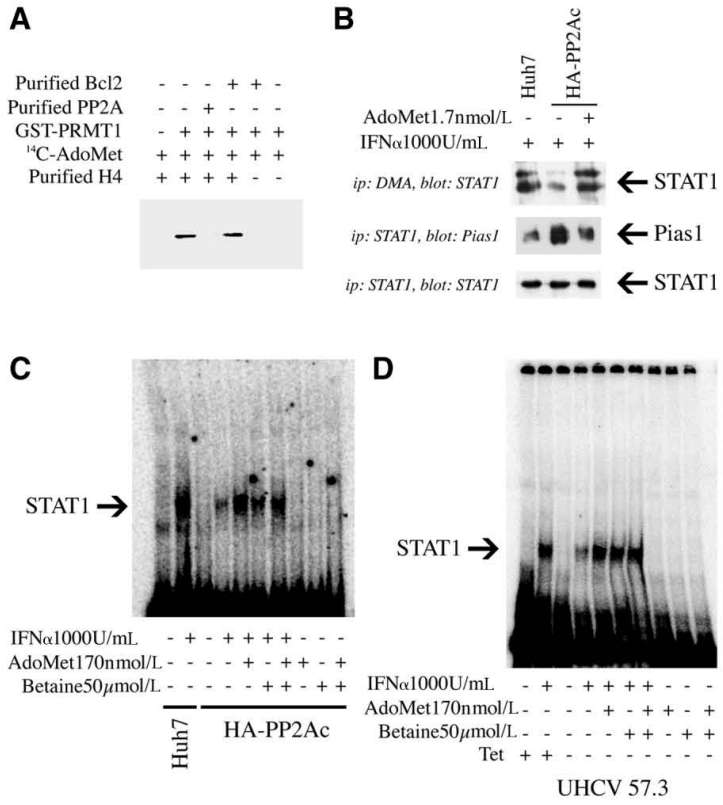

Fig. 3. The inhibition of protein arginine methyltransferase 1 (PRMT1) by protein phosphatase 2A catalytic subunit (PP2Ac) can be overcome by AdoMet and betaine. (A) PP2Ac directly interacts with PRMT1 and inhibits its methyltransferase activity. Purified histone H4 can be methylated *in vitro* by purified PRMT1 (GST-PRMT1 fusion protein), lane 2. The addition of purified PP2A completely blocked the enzymatic activity of PRMT1 (lane 3). To control the specificity of the inhibition of PP2A on PRMT1, purified Bcl2 was used and had no effect (lane 4). Lanes 5 and 6 are controls without histone H4. (B) Huh7 cells expressing a constitutive active form of PP2Ac (HA-PP2Ac) have a constant inhibition of PRMT1. In these cells, STAT1 methylation as detected by an IP-Western blot (immunoprecipitation with antibodies to dimethyl-arginine, Western blot with STAT1 antibodies) is diminished (lane 2, upper panel), and binding of PIAS1 to activated STAT1 as detected by a co-IP (immunoprecipitation with STAT1 antibodies, Western blot with PIAS1 antibodies) is enhanced (lane 2, middle panel). A 2-hour pretreatment of HA-PP2Ac cells (lane 3, upper panel) with AdoMet restores STAT1 methylation to normal levels found in Huh7 cells (lane 1, upper panel), and corrects the increased binding of PIAS1 (middle panel). The lower panel shows the loading control. (C) Electrophoretic mobility shift assay (EMSA) using the SIE-m67 oligonucleotide probe. IFNα induced binding of activated STAT1 is impaired in HA-PP2Ac cells compared with the parent cell line Huh7 (lanes 2 and 4). Pretreatment of HA-PP2Ac cells (lanes 5-7) with AdoMet (170 nmol/L, 2 hours) and/or Betaine (50 μmol/L, 2 hours) restores STAT1 shifts to nearly normal levels found in Huh7 cells (lane 2). Lanes 8-10 show controls without IFNα. (D) Improvement of IFNα signaling by AdoMet and betaine in cells expressing HCV proteins. UHCV57.3 cells were cultured in presence of tetracycline (no HCV protein expression, lanes 1 and 2) or without tetracycline for 24 hours (de-repression of HCV protein expression, lanes 3-9). HCV protein expression leads to an inhibition of the STAT1 gel shift (lane 4). A 2-hour pretreatment of de-repressed cells expressing HCV proteins with AdoMet or/and betaine (lanes 5-7) restores STAT1 DNA binding to normal levels found in cells without HCV protein expression (lane 2). Lanes 8-10 show controls. AdoMet, S-adenosyl-L-methionine.

pretreatment with AdoMet and betaine. Both in HA-PP2Ac cells and in UHCV57.3 cells expressing HCV proteins, the induction of IP-10 was impaired. In both cell lines, IP-10 induction could be restored by treating cells with AdoMet and betaine (Fig. 5).

AdOx Inhibits STAT1 DNA Binding. If an increased methylation of STAT1 after treatment with AdoMet and betaine improves IFNα signaling, then one would suppose that the inhibition of STAT1 methylation also inhibits the binding of activated STAT1 in a gel shift assay. We therefore treated Huh7 cells and HA-PP2Ac cells with adenosine dialdehyde (AdOx). AdOx is an inhibitor of S-adenosylhomocysteine hydrolase, and treatment of cells with AdOx leads to a marked intracellular accumulation of S-adenosyl-L-homocysteine (AdoHcy) (Fig. 4).[35-38] AdoHcy is a competitive inhibitor of PRMT1 and other methyltransferases. When cells were treated with AdOx, we

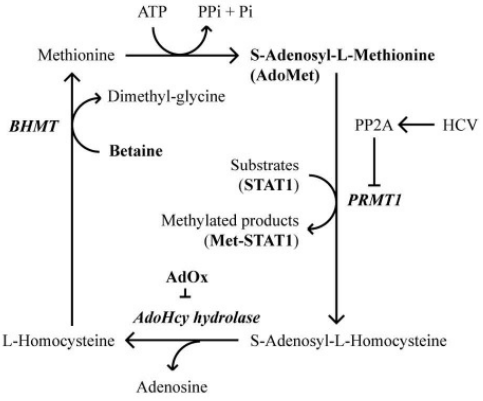

Fig. 4. Proposed model for the role of the AdoMet cycle in the chronic HCV infection. Infection by HCV leads to an upregulation of PP2Ac in liver cells. PP2A inhibits PRMT1. PRMT1 catalyzed STAT1 methylation is reduced. Un-methylated STAT1 is a less efficient inducer of IFNα stimulated genes. This deficiency of IFNα signal transduction can be corrected by increasing the intracellular concentration of AdoMet (shifting the equilibrium of PRMT1 catalylized methylation reaction to the right). AdoMet can be increased by adding the substance itself or by adding betaine. Betaine is the methyl group donor for the conversion of L-homocysteine to methionine, a direct precursor of AdoMet., betaine homocysteine methyltransferase. AdoHcy, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine.

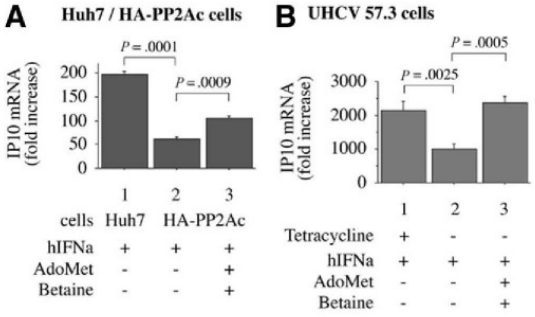

Fig. 5. AdoMet and betaine improve IFNα induced gene expression-.Huh7 and HA-PP2Ac cells (A) and UHCV57.3 cells (B) were stimulated with hIFNα (1,000U/mL) for 6 hours with or without a pretreatment with AdoMet (170 nmol/L) and Betaine (50 μmol/L) for 18 hours. The induction of the interferon target gene IP10 was measured with real-time RT-PCR. Samples were done at least in triplicate. Shown are the mean values and the standard errors of the mean. The *P* values were obtained using the ANOVA test. (A) After treatment with IFNα, the transcriptional induction of IP10 is inhibited in HA-PP2Ac cells (lane 2) compared with the parent cell line Huh7 (lane1). AdoMet and Betaine can restore the response to IFNα even in the presence of PP2Ac over-expression (lane 3). (B) UHCV57.3 cells were cultured either with tetracycline to suppress the expression of HCV proteins (= control, lane 1) or without tetracycline for 24 hours to induce the expression of HCV proteins (lane 2 and 3). Treatment of de-repressed cells with IFNα alone reduced the transcriptional induction of IP10 about 4 fold (lane 2). When cells were pretreated with AdoMet and Betaine, IP10 induction by IFNα was significantly improved (lane 3). AdoMet, S-adenosyl-L-methionine.

observed a time-dependent inhibition of STAT1 DNA binding in gel shift assays.(Fig. 6).

AdoMet and Betaine Increase the Antiviral Effects of IFNα. We then wanted to test if AdoMet and betaine could improve the antiviral efficiency of IFNα. Huh7 cells harboring a subgenomic HCV replicon are a well-established model for HCV replication.[19] In these cells, HCV subgenomic RNA replication can be efficiently inhibited by IFNα in a time- and dose-dependent way.[39] Interestingly, we could increase the effect of IFNα about tenfold by adding AdoMet and betaine to IFNα (Fig. 7A). The combination of 100 IU/mL IFNα plus AdoMet and betaine achieved the same reduction in replicon RNA as a monotherapy with a tenfold higher dose (1000 IU/mL) of IFNα. We then confirmed the increase of the antiviral efficacy of IFNα by AdoMet and betaine in a second experimental system (an overview of the experiment is shown in Supplementary Fig. 2). UHCV57.3 cells were first cultured for 24 hours without tetracycline to induce HCV protein expression (and inhibition of IFNα signaling). During the last 3 hours, AdoMet and betaine were added. They were then infected with vesicular stomatitis virus (VSV) and at the same time treated with IFNα alone or in combination with AdoMet and betaine. We hypothesized that the combination treatment would inhibit the replication of VSV in these cells more efficiently than the monotherapy. The supernatant of these cultures was then used to infect Vero cells, and VSV replication was

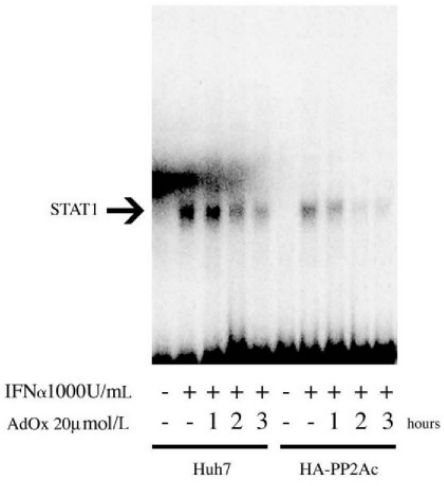

Fig. 6. Inhibition of PRMT1 by Adox inhibits IFNα induced Jak-STAT signaling. EMSA with m67 probe showing a time dependent inhibition of the STAT1 gel shift intensity by AdOx, an inhibitor of PRMT1. AdOx, adenosine dialdehyde.

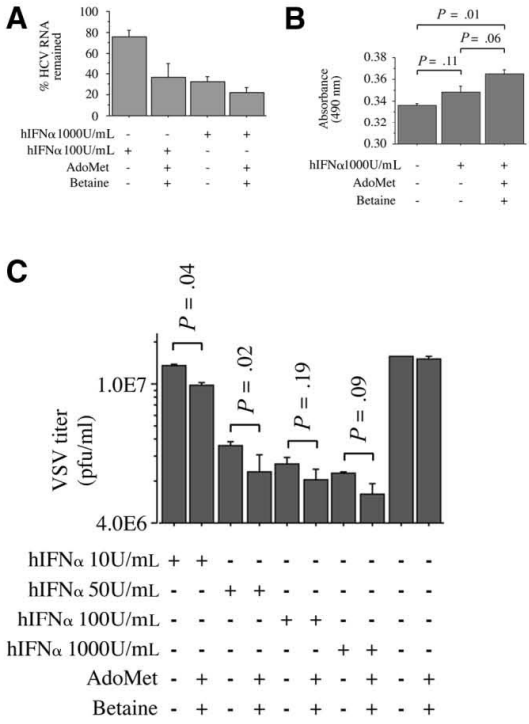

Fig. 7. Impact of AdoMet and betaine on antiviral effects of IFNα. (A) AdoMet and betaine increase the inhibitory effect of IFNα on the replication of a HCV replicon. Huh7 harboring HCV replicon cells were treated for 18 hours with 1000 IU/mL (lanes 1 and 2) or 100 IU/mL IFNα (lanes 3 and 4) without (lanes 1 and 3) or with 170 nmol/L AdoMet and 50 μmol/L betaine (lanes 2 and 4). The amount of replicon RNA was measured by real-time RT-PCR, and calculated as percentage of the baseline value. Mean values from 3 independent experiments are shown. Error bars show standard error of the mean. Of note, the addition of AdoMet and betaine to 100 IU/mL IFNα (lane 2) resulted in the same amount of replicon RNA reduction as a monotherapy with 1000 IU/mL (lane 3). (B) Vero cell viability after infection with supernatant from VSV infected cells. UHCV57.3 cells were first cultured in the absence of tetracycline to induce HCV protein expression. During the last 3 hours, AdoMet (170 nmol/L) and betaine (50 μmol/L) were added. They were then infected with 10pfu VSV and at the same time left untreated (lane 1), treated with 1000 IU/mL IFNα (lane 2), or treated with IFNα and AdoMet (170nmol/L) and betaine (50 μmol/L) (lane 3). After 24 hours incubation, 15 μL of the supernatant were used to infect 30,000 Vero cells. Viability of Vero cells was then tested 24 hours later using a colorimetric method for determining the number of viable cells. Shown is the mean absorbance at 490nm from two duplicate samples (± SEM). Fisher's *t* test was used for statistical comparison of the groups. *P* values are indicated. The combination treatment significantly improved cell viability compared with no treatment. (C) Betaine and AdoMet potentiate the antiviral effect of hIFNα. UHCV57.3 cells were cultured in the absence of tetracycline to induce the expression of HCV proteins. They were infected with VSV and treated with different concentrations of hIFNα (10, 50, 100, or 1000U/mL) as indicated, either alone or in combination with 170 nmol/L AdoMet and 50 μmol/LM Betaine for 24 hours. At every concentration of hIFNα tested, the combination treatment inhibited the viral replication more effectively than the monotherapy. The experiments were done in triplicate. Fisher's *t* test showed statistically significant differences for the samples treated with 10 or with 50 IU/mL hIFNα. AdoMet, S-adenosyl-L-methionine; VSV, vesicular stomatitis virus.

quantified using a cell viability assay. In this assay, cell viability is inversely correlated with the viral titer. Compared with no treatment (Fig. 7B, lane 1), IFNα monotherapy did not significantly improve cell viability (Fig. 7B, lane 2), whereas the combination treatment (Fig. 7B, lane 3) significantly inhibited viral replication in UHCV57.3 cells even in the presence of HCV proteins. Finally, we confirmed these results using a plaque assay (Fig. 7C).

Discussion

Our analysis of 96 biopsy samples from patients with CHC and of 25 control samples provided solid evidence that the expression level of PP2Ac is increased during CHC infection. PP2Ac is an abundant protein that is involved in many signaling pathways, and its expression level and activity are tightly regulated.[40,41] It has been estimated that PP2Ac accounts for 0.3% to 1.0 % of total cellular proteins.[42] We found a median increase from 4.5 to 6.5 ng/μg total protein in liver biopsy extracts from patients with CHC compared with controls. Interestingly, the median expression of PP2Ac in a subgroup of patients infected with the difficult-to-treat genotype 1 was even higher (7.8 ng/μg total protein). This further supports our hypothesis that overexpression of PP2Ac is involved in resistance to IFNα treatment. Given the fact that PP2Ac is a very abundant cellular protein, a 50% increase of its expression level is most likely biologically significant, and will have multiple effects on several intracellular signaling pathways.

In the present study, we concentrated our analysis on an aspect of PP2A biology that has not yet been thoroughly studied, *i.e.*, its role in the regulation of PRMT1. We describe here that PP2Ac can directly bind to and inhibit PRMT1 in an *in vitro* methylation assay with purified proteins. Of note, PRMT1 was involved in many methylation reactions, and an inhibition of its catalytic activity by PP2Ac had profound consequences for many cellular pathways. Most interesting for IFNα signaling is the role of PRMT1 in STAT1 methylation. STAT1 arginine methylation was shown to be important for its reversible association with PIAS1. PIAS1 binds to unmethylated STAT1 and inhibits the DNA binding of STAT1 even after its activation by tyrosine phosphorylation.[14] Recently, the role of STAT1 methylation has been disputed in two reports.[43,44] In both reports, no conclusive evidence for STAT1 methylation could be found. In contrast to these reports and in accordance with the report by Mowen et al.,[14] we consistently found in our experimental systems that the manipulation of STAT1 methylation had consequences on DNA binding of STAT1 and the induction of IFNα target genes (ISGs).

It has been shown that only a subset of ISGs is regulated by PIAS1-STAT1 interactions.[13] One of the better-described PIAS1 dependent ISGs is IP-10, and we therefore analyzed its induction by real-time RT-PCR analysis. We found an inhibition of IP-10 induction in cells overexpressing PP2Ac and in cells that express HCV proteins (Fig. 5). In UHCV57.3 cells that expressed HCV proteins, AdoMet and betaine could restore the normal IP-10 induction by IFNα, and in HA-PP2Ac cells IP-10 induction by IFNα was significantly improved by AdoMet and betaine. Because of the pleiotropic effects of AdoMet and betaine on cells, we can not exclude that methylation of proteins other than STAT1 could be involved in the improvement of IFNα induced target gene induction. However, as shown in Fig. 3, AdoMet and betaine treatment can increase STAT1 methylation, can reduce STAT1-PIAS1 association, and can improve STAT1 DNA binding. We therefore conclude that at least in part, the improved IFNα target gene induction after AdoMet and betaine treatment is mediated by an enhanced DNA binding of STAT1.

We then tested the effect of AdoMet and betaine on the biological activity of IFNα in HCV replicon cells and in UHCV57.3 cells. Strikingly, the combination treatment was about tenfold more potent for inhibiting replication of HCV replicons than the IFNα monotherapy. This effect was consistently found when the replicon RNA levels were analyzed after 18 hours of treatment. Most HCV replicons are very sensitive to IFNα, and longer treatments for 48 hours or 72 hours result in a profound suppression of replicon RNA levels even when low concentrations of IFNα (*e.g.*, 100 IU/mL or 10 IU/mL) are used.[39,45,46] Because of this high efficacy of IFNα, we found no significant difference when adding AdoMet and betaine in 48-hour or 72-hour treatments (data not shown). We therefore confirmed the effect of AdoMet and betaine in a second system using UHCV57.3 cells infected with VSV (Fig. 7B-C). In these experiments, the addition of AdoMet and betaine also significantly improved the antiviral efficacy of IFNα. The absolute changes in terms of virus titers are not very large (Fig. 7C, logarithmic scale), but often large enough to match the efficacy of the next higher IFNα concentration in this dose response curve. We do not know if the observed improvement of the antiviral efficacy of IFNα (albeit statistically significant) would make a difference in the context of a pegIFNα plus ribavirin treatment of CHC. This will have to be tested in clinical trials.

Over the last years, major progress has been made in the understanding how HCV interferes with the induction of IFNs in infected cells. It has been found that HCV NS3/4A protease inhibits the phosphorylation of IFN regulatory factor 3 (IRF-3), a transcription factor central for the induction of IFNβ.[47] Further studies identified RIG-I, a double strand RNA sensor protein, as the sensor of HCV RNA and inducer of IFNβ.[47,48] Recently, the adaptor protein that couples RIG-I to the downstream kinases IKKϵ and TBK1 has been identified and named CARDIF, MAVS, VISA and IPS-1 by four independent groups.[49-52] HCV NS3/4 protease can cleave and inactivate Cardif, thereby blocking the induction of IFNβ.[52] These findings may explain how HCV can inhibit the activation of the IFN system and establish a persistent infection in a majority of patients. On the other hand, the inhibition of the RIG-I – IRF3 pathway can not readily explain why so many patients do not respond to a therapy with pegIFNα and ribavirin. In this therapeutic setting, the IFN system does not need to be activated through the induction of endogenous IFNβ and IFNαs. The failure of IFN based therapies has to be caused either by a block in the signaling pathway or by blocking of IFN effector systems. We have provided evidence in support of the former, and we show here that at least in cultured cells, the block in IFNα signaling can be alleviated by a co-treatment with AdoMet and betaine.

There are potential clinical implications of these results. Taken together, our results suggest the addition of AdoMet and betaine to the current standard treatment of CHC with pegIFNα and ribavirin could increase the efficacy of the treatment. This hypothesis will have to be tested in clinical trials. In our experiments we used concentrations of AdoMet (170 nmol/L) and betaine (50 μmol/L) that can be obtained in the serum of patients by oral application of these substances in nontoxic doses. For example, after a single oral dose of 400mg AdoMet, peak serum concentrations of 1 to 2 μmol/L are obtained,[53] about 6 to 12 times more than the concentrations used in our experiments. In another study, steady state serum concentrations after multiple oral doses (2 $\times$ 800 mg/d) over 4 weeks were 790 nmol/L.[54] AdoMet and betaine are both nontoxic substances that are available in many countries without prescription, and it might be tempting to add them to the current standard therapy with pegIFNα and ribavirin. However, we would advise to use these substances only in the context of well-designed clinical studies.

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3.3 Inhibition of Interferon alpha Signaling by Hepatitis B Virus

Running title: HBV Interference with Interferon alpha Signaling

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Abstract

Interferon alpha (IFNα) and pegylated IFNα (pegIFNα) are used for the treatment of chronic hepatitis B (CHB). Unfortunately, only a minority of patients can be cured. The mechanisms responsible for HBV resistance to (peg)IFNα treatment are not known. PegIFNα is also used to treat patients with chronic hepatitis C. As with chronic hepatitis B, many patients with chronic hepatitis C (CHC) cannot be cured. In CHC, IFNα signaling has been found to be inhibited by an upregulation of protein phosphatase 2A (PP2A). PP2A inhibits protein arginine methyltransferase 1 (PRMT1), the enzyme that catalyzes the methylation of the important IFNα signal transducer STAT1. Hypomethylated STAT1 is less active because it is bound by its inhibitor PIAS1. In the present work we investigated if similar molecular mechanisms are also responsible for the IFNα resistance found in many patients with chronic hepatitis B. We analyzed the expression of PP2A, the enzymatic activity of PRMT1 (methylation assays), the phosphorylation and methylation of STAT1, the association of STAT1 with PIAS1 (co-immunoprecipitation assays), the binding of activated STAT1 to interferon stimulated response elements (EMSAs), and the induction of interferon target genes (real time RT-PCR) in human hepatoma cells expressing HBV proteins, as well as in liver biopsies from patients with chronic hepatitis B and from controls. We found an increased expression of PP2A and an inhibition of IFNα signaling in cells expressing HBV proteins and in liver biopsies of patients with CHB. The molecular mechanisms involved are similar to those found in chronic hepatitis C.

Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) (19, 21). Chronic hepatitis B (CHB) can progress to cirrhosis and hepatocellular carcinoma. Approved treatments of CHB include a few nucleos(t)ide analogues such as lamivudine and adefovir, or interferon α (IFNα), recently in pegylated form (PegIFNα) (19). PegIFNα2a given for 48 weeks can induce HBeAg seroconversion in 32% of the patients (20). However, over 60% of patients will continue to suffer from chronic active hepatitis B despite PegIFNα2a therapy. The molecular mechanisms responsible for the ineffectiveness of IFNα treatments in CHB are not known. PegIFNα (in combination with ribavirin) is also the current standard therapy for chronic hepatitis C (CHC). Interestingly, as in CHB, PegIFNα is not effective in many patients. Over the last years, several molecular mechanisms responsible for viral evasion of the type I IFN system have been studied (10, 14). One of these mechanisms involved in the evasion of hepatitis C virus (HCV) has been elucidated in our laboratory over the last years: HCV proteins interfere with IFNα induced signaling through the Jak-STAT pathway (3, 8, 15).

The interferon system is an important component of the host response against viruses, and mice with deficiencies of IFN receptors or of signal transducer and activator of transcription 1 (STAT1) are highly susceptible to viral infections (2, 9, 23). IFN-α/β binding to its receptor activates members of the Jak family of tyrosine kinases, which then phosphorylate STAT1, STAT2 and STAT3 on a single tyrosine residue. Phosphorylated STATs form dimers, translocate into the nucleus, bind to promoter elements of interferon stimulated genes (ISGs), and activate the transcription of ISGs (4). This activation cycle is terminated by tyrosine dephosphorylation in the nucleus, followed by the decay of dimers and the nuclear export of STATs (5, 30). The pathway is tightly controlled by a number of inhibitory proteins (18, 27), amongst them protein inhibitor of activated STAT1 (PIAS1) (22). PIAS1 inhibits the last step in the Jak-STAT pathway, i.e., DNA binding. Complex formation between STAT1 and PIAS1 is regulated by an important posttranslational modification of STAT1, arginine methylation (25). Methylation of STAT1 is catalyzed by protein arginine methyltransferase 1 (PRMT1) and protects STAT1 from binding and inactivation by PIAS1 (25).

We have previously reported that HCV inhibits IFN-α induced signaling at the level of STAT DNA binding (3, 15). Expression of HCV proteins in cells induces an increased expression of protein phosphatase 2Ac (PP2Ac) (8). PP2Ac was also overexpressed in extracts from liver cells of HCV transgenic mice and in liver biopsies from patients with CHC (8). PP2A is a heterotrimeric protein phosphatase consisting of a 36-kilodalton catalytic C subunit (PP2Ac), a 65-kilodalton structural A subunit, and a variable regulatory B subunit. PP2A is expressed in all cell types, is primarily a serine/threonine phosphatase, and is involved in a wide range of cellular processes including cell cycle regulation, cell morphology, development, signal transduction, translation, apoptosis and stress response (17, 24). PP2A regulates IFNα signaling through a strong inhibition of PRMT1 (6). Inhibition of PRMT1 results in a reduced level of STAT1 methylation and an increased binding of STAT1 by its inhibitor PIAS1 not only in cultured cells expressing either HCV proteins or overexpressing PP2Ac, but also in liver extracts of HCV transgenic mice and in liver biopsies from patients with CHC (8).

In the present paper, we have used a cell line that allows the controlled expression of hepatitis B virus, and liver biopsies from patients with CHB to analyze PP2Ac expression and IFNα signaling through the Jak-STAT pathway. Although HCV and HBV are completely unrelated viruses, we found very similar molecular mechanisms of viral interference with IFNα signaling.

Materials and Methods

Reagents, Antibodies, and Cells.

Human IFNα (Roferon) was obtained from Hoffmann LaRoche (Basel, Switzerland). Purified PP2A and anti-PP2Ac were purchased from Upstate (LucernaChem, Luzern, Switzerland). Anti Phospho-STAT1 (Tyr 701) was purchased from Cell Signaling Technology (BioConcept, Allschwil, Switzerland). Anti-STAT1 was from SantaCruz (SantaCruz Biotechnology, Inc, Heidelberg, Germany). Monoclonal antibody to methyl and dimethyl arginine was purchased from Abcam (Abcam Limited, Cambridge, United Kingdom). ^{14}C-AdoMet (specific activity 53 mCi/mmol) was obtained from Amersham Biosciences (Amersham Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland). C-terminally truncated recombinant HBV core protein (amino acid sequence 1-149), used as immunoblot standard, was purified as previously described (1). For detection, the anti-HBV core protein mouse monoclonal antibodies mc312 and mc158 (26) were used. H7TA-61 (a gift of Darius Moradpour) are Huh7 cells that constitutively express the tetracycline-controlled transactivator, tTA (13). H7TA-61 cells were then transfected with HBV expressing plasmid pTRE-HBVT, and stable clones were selected as described elsewhere (29). In brief, pTRE-HBVT contains a slightly overlength HBV genome (subtype ayw; (11)) fused behind a Tet-response element (TRE) controlled minimal promoter. In the absence of tetracycline or its analogue doxycycline (Dox), tTA binds to and activates the promoter, generating authentic HBV pregenomic RNA, the template for translation of the viral core and polymerase proteins, and substrate for packaging into, and reverse transcription inside, viral core particles. In the presence of Dox, pregenomic RNA transcription is suppressed to below detectability. Because the mRNAs for the surface proteins (L, M, and S) and X protein are transcribed from the endogenous HBV promoters they are not subject to Dox control, allowing for an essentially constitutive expression of the gene products. From one of the clones displaying tight control by Dox, the stable cell line Huh7.93 was established and used in this study.

Patients and Biopsies.

From August 2002, to April, 2005, all patients with chronic hepatitis B referred to the outpatients liver clinic of the University Hospital Basel who had a liver biopsy were asked for their permission to use part of the biopsy for this study. The protocol was approved by the ethical commission of Basel. Written informed consent was obtained from all patients who agreed to participate in the study. A semiquantitative grading and staging of all biopsies of patients with chronic hepatitis B was done according to Ishak (16). The expression of HBsAg and HBcAg was analyzed by routine immunohistochemistry methods in the Institute of Pathology of the University of Basel, and the expression levels were semiquantitatively determined as percentage of

hepatocytes with positive stainings. Control samples were from patients who underwent ultrasound-guided liver biopsies of focal lesions (mostly metastasis of carcinomas) and who were asked for their permission to obtain a biopsy from the normal liver tissue outside the focal lesion. Only samples with histologically confirmed absence of liver disease were used as controls.

Preparation of Extracts from Cells and Liver Biopsies.
Whole cell lysates and nuclear extracts were prepared as described (8). The liver biopsies were homogenized in 100 µl of lysis buffer (100 mM NaCl, 50 mM Tris pH 7.5, 1mM EDTA, 0.1 % Triton X-100, 10 mM NaF, 1 mM PMSF, and 1 mM sodium orthovanadate), and the lysates were then centrifuged at 14000 rpm for 5 minutes. Protein concentrations were determined with the BioRad Protein Assay (Bio-Rad Laboratories AG, Reinach, Switzerland).

Immunoprecipitation and Immunoblotting.
Immunoprecipitation and immunoblotting was done as described (8). To measure PP2Ac expression in human liver biopsies and in hepatoma cells, 50, 100, and 200 ng of purified PP2Ac was loaded on each gel. These 3 samples allowed the calculation of a standard curve for each gel. The intensity of each band was measured by densitometry analysis using NIH Image software. The amount of PP2Ac in each liver biopsie and in each hepatoma cell sample was then calculated according to the standard curve.

Electrophoretic Mobility Shift Assay.
EMSAs were done as described (15) using 1 µg nuclear extracts aliquots and the SIE-m67 oligonucleotide probe . STAT1 was supershifted with antibody sc-346 from Santa Cruz (LabForce AG, Nunningen, Switzerland).

RNA isolation, reverse transcription, and SYBR-PCR.
Total RNA was isolated from the cells using a Perfect RNA Eukaryotic Mini kit (Eppendorf, Vaudaux-Eppendorf, Basel, Switzerland) according to the manufacturer's instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega, Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Promega) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70°C and then for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. SYBR-PCR was performed based on SYBR green fluorescence (SYBR green PCR master mix, Applied Biosystems, Foster City, CA). To prevent genomic DNA amplification, the primers for GAP-DH and IP10 were designed across exon-intron junctions. The primers for GAPDH were 5` GCTCCTCCTGTTCGACAGTCA 3` and 5`ACCTTCCCCATGGTGTCTGA 3`. The primers for IP10 were 5` CGATTCTGATTTGCTGCCTTAT 3`and 5` GCAGGTACAGCGTACGGTTCT 3`. The ΔCT value was derived by subtracting the threshold cycle (CT) value for GAPDH, which served as an internal control, from the CT values for IP10. All reactions were run in duplicate by use of an ABI 7000 sequence detection system (Applied Biosystems). mRNA expression levels of IP10 was expressed as a fold increase according to the formula 2ΔCT(PBS)-ΔCT(Interferon-stimulation) .

Methylation Assay.
To compare methyltransferase activity between Huh7 cells and Huh7.93 cells 20 µg of whole-cell lysate from each cell line were incubated in the presence of 3 µl of 14C-AdoMet for 2 h at 37°C. The reactions were then stopped by adding 5 µl of sample loading buffer, and the reaction volumes were boiled for 5 min and separated on an 8% SDS-polyacrylamide gel. The upper part of the gel was dried and then exposed to Phosphorimager plate for 3 days. The lower part of the gel was cut out and stained with Coomassie blue to check for equal loading.

Quantification of HBs antigen in the culture medium of Huh7.93 cells
Huh7.93 cells were grown for up to 7 days in DMEM containing doxycycline. 500µl of the culture medium was sampled daily, and the amount of HBsAg quantified using the automated test system Elecsys 2010 for Hoffmann LaRoche (Basel, Switzerland) (32). As a negative control sample, culture medium was used. In short, Elecsys 2010 is a two-step sandwich assay developed and routinely used for the quantative detection of HBsAg in human serum or plasma.
In the first incubation step, biotinylated and ruthenylated monoclonal antibodies directed against HBsAg used to detect HBsAg in the sample. Streptavidin-coated magnetic microparticles are then added to the mixture. In the measuring cell of the Elecsys 2010 system, the microparticles are magnetically captured on the surface of the electrode. Unbound substances are removed with ProCell. Application of voltage to the electrode induces chemiluminescence, which is measured with a photomultiplier.
Results are calculated with the Elecsys software by comparing the chemiluminescence signal obtained from the sample with the cutoff value previously obtained by HBsAg calibration. The amount of HBsAg in the samples is then expressed as the signal/cutoff ratio (s/co).

Immunofluorescence with Huh7.93 and H7TA61 cells.
Huh7.93 and H7TA61 cells were grown in a 6 well plate until 90% confluence. After aspirating out the culture medium, the wells were washed once with PBS. To fix the cells, 100% methanol (-20°C) was added to the cells for 10 min at -20°C. After another 3 washes for 5 min at RT with Tris-buffered saline tween-20 (TBST), the cells were incubated with blocking solution (TBST with 5% BSA) for 1h at RT. After that the cells were washed once with TBST, before incubation with anti-Hepatitis B virus surface antibody (catalog No MU364-UCE, BioGenex, San Ramon, CA, USA) overnight at 37°C. After 3 washes with TBST, they were incubated with Cy3-conjugated secondary antibody (Amersham, Dübendorf, Switzerland) for 1 hour and 30 minutes at room temperature. Nuclear staining was performed with Hoechst (Amersham) for 5 minutes at room temperature. After washing, a coverslip was mounted in FluorSave Reagent (Calbiochem) and pictures were taken under the microscpope.

Results

Expression of hepatitis B virus antigens in Huh7 cells induces expression of PP2Ac.

To investigate if HBV interferes with IFNα signaling we used the controllably HBV producing cell line Huh7.93 (D. Sun and M. Nassal, manuscript in preparation) and compared it to unmodified Huh7 cells and, selectively, to the parental tTA containing H7TA-61 cells. The Huh7.93 cell line is one of several lines established by stably transfecting the HBV expression plasmid pTRE-HBVT (see Fig. 1A, and Materials and Methods for details) into the Huh7 derived cell line H7TA-61 which contains the Tet-controlled transactivator, tTA. In the absence of tetracycline or its analog doxycycline (Dox), tTA activates the TRE promoter, leading to transcription of HBV pregenomic RNA (pgRNA), and consequently core and polymerase protein translation; under these conditions, viral replication occurs and complete virions are generated. In the presence of Dox, transcription of pgRNA is suppressed such that no viral replication can be detected by Southern blotting (D. Sun and M. Nassal, manuscript in press, Journal of Hepatology). In contrast, the surface proteins, and likely the HBx protein, are constitutively expressed from the original endogenous viral promoters which are not Dox responsive. These data were corroborated here in that HBsAg was easily detected by immunostaining in the perinuclear cytoplasma of Huh7.93 cells (Fig. 1B) and in the supernatants of Huh7.93 cells cultured with Dox (Fig. 1C) whereas core protein became detectable only when the cells were cultured in medium without Dox (Fig. 1D).

Figure 1

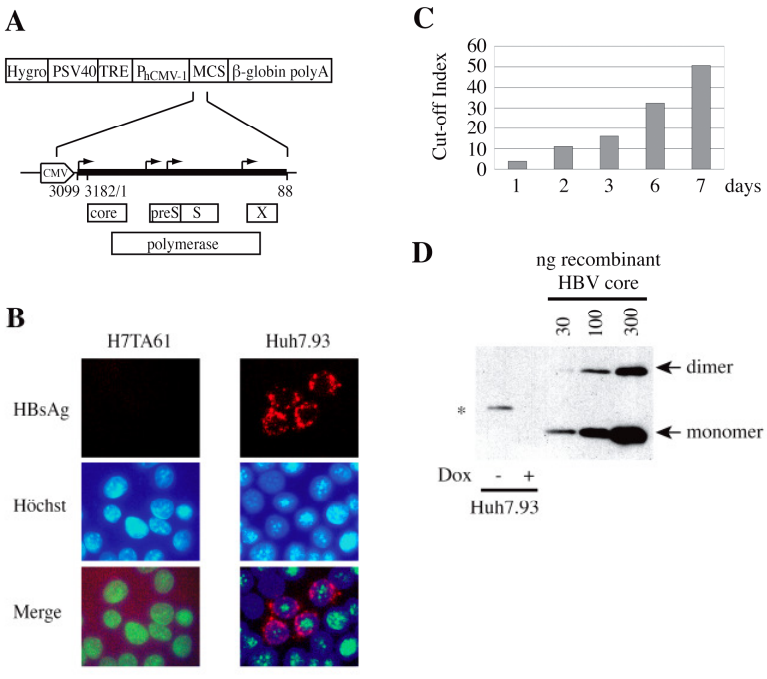

FIGURE 1

(A) Schematic map of plasmid pTRE-HBVT used to establish the HBV expressing Huh7.93 cell line. A slightly overlength (1.05 x) HBV genome was cloned behind the Tet-Response-Element/minimal CMV promoter in plasmid pTRE2hyg (Clontech) such that transcription of the full-length pregenomic RNA of

HBV is controllable by the presence or absence of Dox. Without Dox, pregenomic RNA and its gene products core and polymerase are generated, leading to viral replication. With Dox, pregenomic RNA transcription is suppressed whereas the subgenomic mRNAs for the surface proteins and X protein are transcribed from the Dox-independent endogenous viral promoters. Arrowheads denote approximate RNA start sites. For authentic poly-adenylation, the genuine viral poly-adenylation signal is contained in the 3´ terminal redundancy of the inserted HBV genome. The position of the HBV genes are indicated by boxes.
(B) HBs antigen is expressed in the perinuclear cytoplasm in Huh7.93 cells.
H7TA61 (left panel) and Huh7.93 (right panel) were stained for HBsAg expression. The cell nuclei were stained with Hoechst dye (middle panels). The merge of the two pictures shows the cytoplasmic location of the HBsAg.
(C) Huh7.93 cells constitutively express HBsAg. Cells were grown in culture medium supplemented with doxycycline. The culture supernatant was sampled at the indicated time points, and HBsAg was quantified on an automated Elecsys 2010 system. The amount of HBsAg is expressed as signal/cutoff ratio. The cutoff ratio was determined with an HBsAg standard provided by the manufacturer.
(D) De-repression of Huh7.93 cells induces expression of HBV core protein. Example of a Western blot showing the samples with 30 ng, 100 ng and 300 ng of recombinant truncated HBV core protein (amino acids 1 to 149), which appears as a monomer of 16/17 kDa and a dimer of 32-34 kDa, and lysates of Huh7.93 cells grown for 6 days without Dox (lane 1) or with Dox (lane 2). The asterisk indicates the position of the 21 kDa full-length core protein in the cells.

Because of its role in regulating the IFN response we first measured the protein expression levels of PP2Ac in Huh7.93 vs. Huh7 or H7TA-61 cells, using purified PP2Ac for calibration (Fig. 2A). In Huh7.93 cell lysates, the mean PP2Ac expression level was 30.2 ng/µg total protein, with a standard error of 3.5 ng/µg (Fig. 2B). This was significantly higher than in Huh7 cell lysates, where the mean PP2Ac expression level was 16.1 ng/µg total protein (standard error 2.2 ng/µg). Inducing HBV replication by culturing the cells without Dox did not further increase the expression level of PP2Ac (Fig. 2C). PP2Ac expression was also significantly increased in Huh7.93 cells when compared to H7TA-61 cells (Fig. 2A). Hence the increased PP2Ac level in Huh7.93 cells is most likely due to the expression of the HBV surface and/or X proteins.
Because PP2Ac can inhibit the enzymatic activity of PRMT1 (6), up-regulation of PP2Ac expression in Huh7.93 cells should inhibit PRMT1 in these cells. We tested PRMT1 activity in cell extracts from Huh7 and from Huh7.93-1 cells using an *in vitro* methylation assay, and found a strong inhibition of PRMT1 in Huh7.93 cells (Fig. 2D).

Figure 2

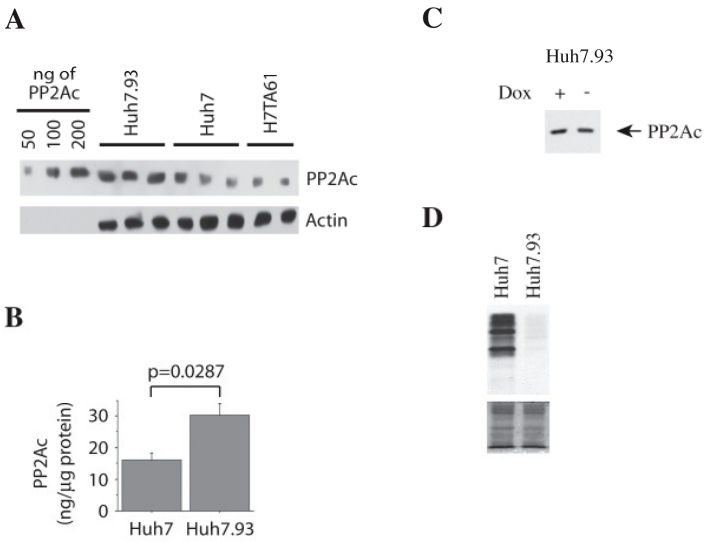

FIGURE 2
(A) Expression of PP2Ac in human hepatoma cells expressing HBV proteins. Western blot showing PP2Ac standard samples with 50 ng, 100 ng and 200 ng of purified PP2Ac in lanes 1-3 (used to generate a calibration curve) and 3 samples each of Huh7 and Huh7.93 cell lysates, as well as 2 samples of H7TA-61 cells (5 µg total protein in each lane). The lower panel is a loading control showing the house keeping protein actin.
(B) Graph of quantified PP2Ac expression levels in Huh7 versus Huh7.93 cells. Shown is the mean of three independent samples with error bars representing the standard errors of the means. The difference in the expression of PP2Ac between control cells and HBV proteins expressing cells is statistically significant ($p = 0.0287$, Mann-Whitney U test).
(C) Huh7.93 cells were grown in medium with and without doxycycline as indicated. PP2Ac expression was measured by Western blot. No further increase of PP2Ac expression was observed in de-repressed cells.
(D) PRMT1 activity is inhibited in Huh7.93 cells. 20 µg of whole-cell lysate from each cell line were incubated in the presence of 3 µl of 14C-AdoMet for 2 h at 37°C. The reactions were then stopped by adding 5 µl of sample loading buffer, and the reactionmixtures were boiled for 5 min and the proteins were separated on an 8% SDS-polyacrylamide gel. The upper part of the gel was dried and then exposed to Phosphorimager plate for 3 days. The lower part of the gel was cut out and stained with Coomassie blue to check for equal loading.

IFNα signal transduction is inhibited in HBV antigen expressing cells.

We then tested if HBV protein expression and PP2Ac overexpression inhibits IFNα signaling. Activated STAT1 forms dimers, translocates into the nucleus and binds to promoter elements of interferon stimulated genes (ISGs). We analyzed binding of activated STAT1 dimers by electrophoretic mobility shift assays (EMSA) with the m67-SIE oligonucleotide probe. A strong gel shift signal was induced by IFNα in Huh7 cells and in H7TA-61 cells, whereas the signal was weak in Huh7.93 cells (Fig.3A). De-repression of Huh7.93 cells by removing Dox from the culture medium did not further decrease the gel shift signal, indicating that the expression of the surface proteins and/or

the X protein is sufficient to impede IFNα signaling (Fig. 3B). This inhibition of STAT1-DNA binding was not caused by a reduced STAT1 expression level, because STAT1 specific bands in Western blots were equally strong in extracts from Huh7 and Huh7.93 cells (Fig. 3C). We also observed no difference in IFNα induced phosphorylation of STAT1 on tyrosine 701 (Fig. 3C). Therefore, we conclude that the inhibition of STAT1 signaling has to be downstream of STAT1 activation at the receptor kinase complex.

Figure 3

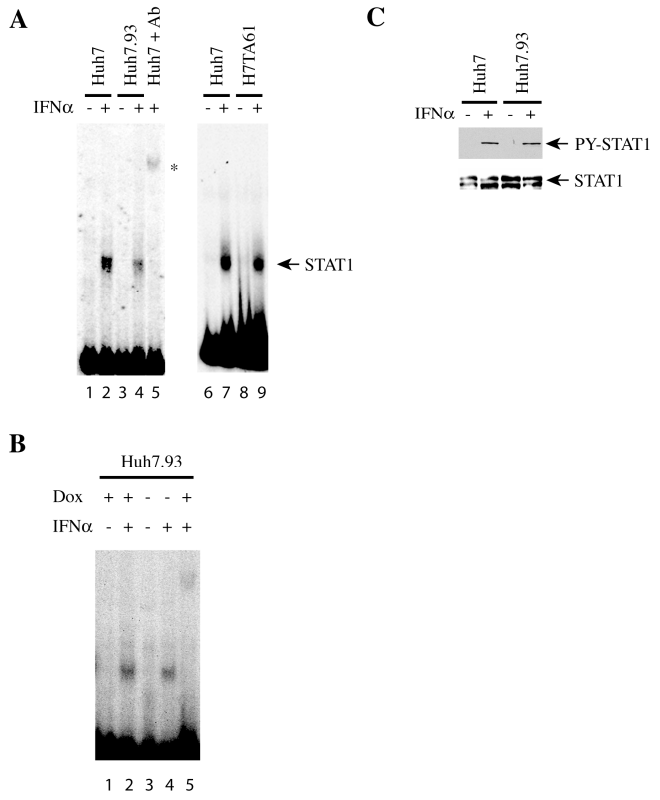

FIGURE 3
(A) IFNα induced binding of activated STAT1 is impaired in Huh7.93 cells compared to the parental cell lines Huh7 and H7TA-61. Electrophoretic mobility shift assay (EMSA) using the SIE-m67 oligonucleotide probe. Cells were left untreated (lane 1,3, 6 and 8) or they were treated for 20 minutes with 1000 U/ml hIFNα (lane 2, 4, 5, 7 and 9). In lane 5, the nuclear extract of Huh7 (hIFNα treated) was incubated with anti-STAT1 antibody prior to the binding reaction to perform a supershift (asterisk).
(B) Huh7.93 cells were cultured in medium with or without Dox as indicated. Cells were then stimulated for 20 min with 1000 U/ml hIFNα. Nuclear extracts were analysed with EMSA with SIE-m67. No further decrease in signal intensity was observed in de-repressed cells.
(C) Cytoplasmatic extracts were used to perform Western blot analysis. There is no difference in the phosphorylation of STAT1 on tyrosine 701 between the control cells and the HBV expressing cells (upper part). Membrane was reblotted for STAT 1 as loading control (lower part).

We next looked at the methylation status of STAT1, and found indeed a strong reduction in Huh7.93 cells (Fig. 4A). Since STAT1 methylation regulates the association of STAT1 and PIAS1 (25), we did co-immunoprecipitation experiments with STAT1 and PIAS1 antibodies. In accordance with the reduced STAT1 methylation, we detected an increased

binding of PIAS1 to STAT1 in Huh7.93 cells (Fig. 4B). Finally, the induction of the interferon stimulated gene IP10 was quantified by measuring the IP10 mRNA concentration after a 6 hour stimulation of cells with IFNα in Huh7 and Huh7.93 cells. The inhibition of the Jak-STAT pathway in Huh7.93 cells on the level of STAT1 methylation resulted in a significantly lower induction of IP10 (Fig. 4C).

Figure 4

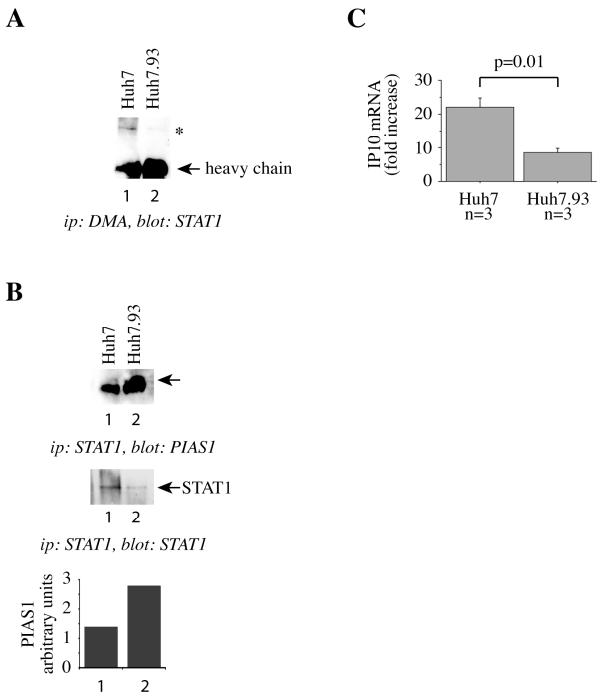

FIGURE 4
(A) STAT1 methylation (asterisk) detected by an IP-Western blot (immunoprecipitation with antibodies to monomethyl-dimethyl-arginine, Western blot with STAT1 antibodies) is impaired in cells expressing viral proteins (lane 2) compared to Huh7 (lane 1).
(B) Binding of PIAS1 to STAT1 as detected by a co-IP (immunoprecipitation with STAT1 antibodies, Western blot with PIAS1 antibodies) in Huh7.93 cells is enhanced (lane 2) compared to Huh7 cells (lane 1). Densitometric analysis of the PIAS1 signals is shown in the lower panel. The values are the integrated densities measured with NIH Image software and expressed as arbitrary units .
(C) Reduced IFNα target gene induction in the presence of HBV proteins.
Huh7 and Huh7.93 cells were stimulated with hIFNα (1000U/ml) for 6 hours. The amount of the interferon target gene IP10 was measured with real-time RT-PCR in three independent samples (each sample was measured in duplicate). The induction of IP10 mRNA was calculated as fold increase of the mRNA amounts in IFNα treated samples versus untreated samples. Shown are the mean values and the standard errors of the mean. The p-value was obtained using the ANOVA test. The transcriptional induction of IP10 is inhibited in Huh7.93 cells (right bar) compared to the parental cell line Huh7 (left bar).

Upregulation of PP2Ac in chronic hepatitis B

To corroborate the physiological relevance of the cell line derived data described above, we next measured the PP2Ac expression levels in liver biopsy extracts from patients with chronic hepatitis B and in non-infected control samples, using a semi-quantitative Western blot method (7). The controls were from patients who had an ultrasound-guided biopsy of a focal lesion in the liver. The liver parenchyma outside the lesion was biopsied as well, and if the histological evaluation did not show any liver pathology, the samples were included as controls for the present study. The median PP2Ac concentration was 11.9 ng/µg total protein in controls, and 17.5 ng/µg total protein in biopsies from patients with chronic hepatitis B, a difference that was statistically significant when tested with the Mann-Whitney U test ($p = 0.0003$). Interestingly, the PP2Ac expression level correlated positively and significantly with the percentage of hepatocytes that stained positively for HBsAg (Fig. 5B, $r = 0.565$, $p = 0.021$, Fisher's z-test). HBcAg expression was found in only 4 of the 18 biopsies. The PP2Ac expression level tended to be higher in these HBcAg positive samples, but the small sample size limits the significance of this result (Fig. 5C). There was no correlation between PP2Ac expression level and the degree of inflammation in these biopsies (data not shown).

Figure 5

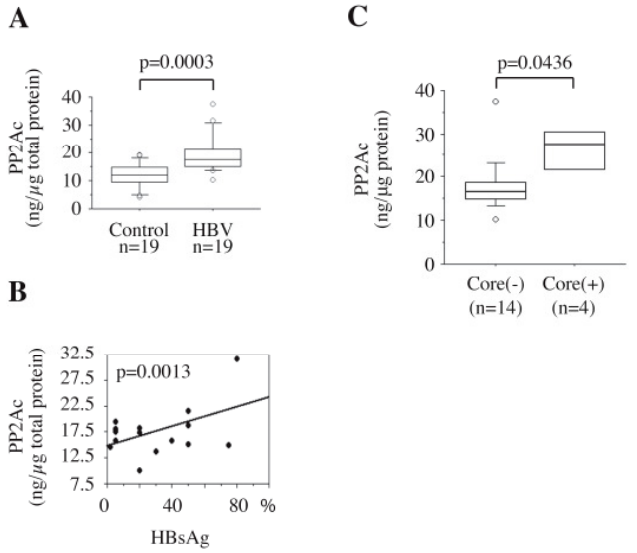

FIGURE 5:

(A) Expression of PP2Ac in human liver biopsies of patients with chronic hepatitis B (CHB).

PP2Ac expression levels were analysed in liver biopsies from patients with CHB and from controls using a semi-quantitative Western blot. On each Western blot gel 50, 100 and 200 ng of purified PP2Ac were loaded to generate a calibration curve. The Western blot signal intensities of these standards and of the samples were measured using NIH Image Software, and the amount of PP2Ac in each sample was calculated according to the calibration curve.

Box-plot diagram of quantified PP2Ac expression levels in liver biopsies of 19 patients with chronic hepatitis B and of 19 control patients without parenchymal liver disease. The difference in the expression of PP2Ac between the two groups is statistically significant (P=0.0003, Mann-Whitney U test).

(B) Correlation between PP2Ac expression and HBsAg expression.

Liver biopsies from patients with CHB were stained for HBsAg expression. The percentage of hepatocytes with positive staining was assessed. The expression of PP2Ac correlated positively with HBsAg expression (r=0.565, z=0.021, Fisher's z-test).
(C) Correlation between PP2Ac expression and HBcAg expression.
HBcAg expression was positive in 4 samples and negative in 14 samples. PP2Ac expression was higher in the group with HBcAg expression. Shown is a boxplot diagram.

Discussion

We have shown previously that hepatitis C virus, a member of the *Flaviviridae* family, induces the upregulation of PP2Ac *in vivo* (liver biopsy studies, HCV transgenic mice) and *in vitro* (tetracycline regulated HCV protein expression, HCV replicon cells) (3, 8). Here we report that HBV, a member of the *Hepadnaviruses*, also induces a significant up-regulation of PP2Ac. PP2A is an important serine/threonine phosphatase involved in many cellular processes (17, 24), and the over-expression of its catalytic subunit PP2Ac is likely to have important consequences for the host cell. We have concentrated on the effect of PP2Ac up-regulation on IFNα signaling through the Jak-STAT pathway. The key finding of these studies was that PP2Ac physically interacts with and inhibits PRMT1 in cells and also in biochemical experiments using purified proteins (6, 8). Here we show that Huh7 cells that express HBV antigens (Huh7.93) have elevated expression levels of PP2Ac compared to generic Huh7 cells as well as the tTA containing H7TA-61 parental cells, and that the enzymatic activity of PRMT1 is strongly inhibited (Fig. 2). Most likely, the inhibition of PRMT1 is a consequence of PP2Ac over-expression. Like PP2A, PRMT1 is an important enzyme expressed in all cells and involved in arginine methylation of many proteins, among them histones (31) and RNA binding proteins such as hnRNPs, fibrillarin, nucleolin and poly(A) binding protein II (12, 28). The arginine methylation of STAT1 by PRMT1 modulates IFNα induced transcription of interferon target genes (25). Methylated STAT1 has a lower affinity to PIAS1, an inhibitor of DNA binding of activated STAT dimers. Because PRMT1 activity is inhibited in HBV antigen expressing cells, STAT1 is present mainly in its unmethylated form (Fig. 4A), and has a higher affinity for PIAS1 as shown in co-immunoprecipitation experiments (Fig. 4B). The increased binding of STAT1 by PIAS1 results in a reduced affinity of STAT1 to its response elements in IFNα target gene promoters, as shown by electrophoretic mobility shift assays (Fig. 3A). Interestingly, we did not observe any substantial differences between Huh7.93 cells grown in the presence vs. absence of Dox. Because the levels of HBV core protein and polymerase are drastically lowered by Dox, the surface proteins and/or the X protein are the most likely candidate antigens responsible for suppressing the IFN response. This question may be answered, for instance, by establishing comparable cell lines in which production of the individual gene products is selectively knocked out. Importantly, the similarly increased PP2Ac levels in liver biopsies from chronic hepatitis B patients vs. the non-infected controls strongly supports the physiological relevance of our cell line data.

In conclusion, we propose that the molecular mechanism by which HBVsuppresses the IFN repsonse involves an up-regulation of PP2Ac as the primary event, with an inhibition of PRMT1 and a reduced STAT1 methylation as its consequence. Unmethylated STAT1 then is bound by PIAS1 and has a reduced capacity to stimulate IFNα target genes. Our

work identifies PP2Ac, PRMT1 and PIAS1 as potential therapeutic targets for strategies aimed at increasing the response rates of IFNα based treatments in chronic hepatitis B.

Acknowledgements

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3.4 Activation of Endoplasmatic Reticulum Stress Response by Hepatitis Viruses Upregulates Protein Phosphatase 2A

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Keywords: Hepatitis C Virus, Hepatitis B Virus, PP2A, endoplasmatic reticulum stress

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Abbreviations:
HCV, hepatitis C virus; HBV, hepatitis B virus; PP2A, protein phosphatase 2A; ER, endoplasmatic reticulum; IFN, interferon; CHC, chronic hepatitis C; STAT, signal transducer and activator of transcription; PIAS1, protein inhibitor of activated STAT1; IRGs, interferon regulated genes; PRMT1, protein arginine methyl transferase 1; UPR, unfolded protein response; ERAD, ER-associated degradation; CREB, cyclic AMP response element binding protein; GFP, green fluorescent protein; ERSE, ER stress response element; CaM kinase, Ca-calmodulin-dependent protein kinase;

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Abstract

The upregulation of protein phosphatase 2 A (PP2A) is an important factor leading to an inhibition of IFNα signaling caused by viral protein expression. Here we describe the molecular mechanism responsible for PP2Ac upregulation by hepatitis C (HCV) and hepatitis B viruses (HBV). Both viruses induce an ER stress response leading to calcium release from the ER and CREB activation, most likely through calcium/calmodulin-dependent protein kinase. CREB binds to a CRE element in the promoter of PP2Ac and induces its transcriptional upregulation. Since PP2Ac is involved in many important cellular processes including cell-cycle regulation, apoptosis, cell morphology, development, signal transduction and translation, its upregulation during ER stress has potentially important implications. Interestingly, PP2Ac upregulation was observed also when ER stress was induced independent from viral protein expression, and therefore might be an integral part of the ER stress response.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma worldwide (1). HCV is a parentally transmitted hepatotropic RNA virus. In the majority of patients HCV evades the early immune response and establishes a persistent infection. Type I interferons (IFNs) are important and potent mediators of the early host response to viral infection, and are also used for the therapy of chronic hepatitis C (CHC). Not surprisingly, therefore, HCV was found to interfere with the induction of IFNs as well as with IFN signaling in infected cells (2). The most important signal transduction pathway for IFNα is the Jak-STAT pathway (3). Signal transducers and activators of transcription (STATs) are activated at the IFN receptor by members of the Jak kinase family through phosphorylation of a single tyrosine residue (4). Phosphorylated STATs form dimers, translocate into the nucleus, and activate IFN target genes through binding to specific response elements in their promoters (5).

We have shown previously that HCV proteins interfere with the IFNα signaling (6, 7). The inhibition is caused by a defective methylation of the essential transcription factor STAT1 (8). Because unmethylated STAT1 is bound by protein inhibitor of activated STAT1 (PIAS1), the binding to the promoters of a subset of interferon regulated genes (IRGs) is abolished, and the IFNα induced antiviral state is partially defective (9, 10). HCV was found to prevent STAT1 methylation through a novel pathway involving the inhibition of protein arginine methyltransferase 1 (PRMT1) by protein phosphatase 2A (PP2A) (8, 11). PP2A overexpression is the key event in this cascade, and indeed, PP2A was consistently found to be overexpressed in cell lines expressing HCV proteins, in liver extracts of HCV transgenic mice, and in liver biopsies of patients with CHC (11, 12).

Recently, we found an induction of PP2A in liver biopsies from patients with chronic hepatitis B (CHB), and in cell lines that express hepatitis B virus (HBV) proteins (13). The finding was surprising because HBV and HCV are not related. We hypothesized that the two viruses induce PP2A overexpression through a common mechanism. Since both viruses use the endoplasmatic reticulum for the production of viral proteins, we investigated if HBV and HCV induce an endoplasmatic reticulum (ER) stress response, and if PP2A upregulation is induced through one or more ER stress signaling pathways.

Cells monitor the physiological load placed on the ER and respond to perturbations in the ER function (i.e. ER stress) by a process known as the unfolded protein response (UPR) (14). The UPR results in the upregulation of ER-resident molecular chaperones such as BiP/GRP78 and GRP94 and thereby augments the folding capacity of the ER. This transcriptional up-regulation is mediated through the activation of the ER resident type I transmembrane kinase and endonucleases IRE1α and IRE1β that differentially splice the mRNA of Xbp-1, an important transcription factor for the activation of UPR target genes (15-18). The UPR also involves the activation of the ER resident type I transmembrane protein kinase PERK and the transcription factor ATF6 (19-21). The cytoplasmic kinase domain of PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2-α). Phosphorylated eIF2-α inhibits the association of mRNA with the ribosomal

60S and 40S subunits and thereby inhibits the translation of proteins. Finally, the UPR also activates the proteasome dependent degradation of proteins through a process called ER-associated degradation (ERAD) (22). All these systems work in a coordinated fashion to improve the efficiency of folding, processing and export of secretory proteins, to reduce the flow of newly translated proteins in the ER, and to remove the fraction of polypeptides that fail to fold correctly.

It is well known that viral infection of a cell can induce ER stress. For HCV it has been published that the expression of envelope E1 and E2 proteins (23, 24), the expression of HCV core proteins (25) or the transfection of subgenomic replicons (26) induce an ER stress response. But there are also publications reporting that HCV disrupts (part of) the ER stress response (27, 28).

In the present work we report that HCV and HBV protein expression in cells induces an activation of ER stress response pathways. Furthermore, pharmacological induction of ER stress with thapsigargin or tunicamycin induced an upregulation and overexpression PP2A. This PP2A induction was dependent on calcium and cyclic AMP response element binding protein (CREB). We propose that the upregulation of PP2A observed in HBV and HCV infections is a consequence of ER stress induced by these viruses.

Materials and Methods

Reagents, Antibodies, and Cells

To induce ER-stress, cells were treated with 1 µM thapsigargin or 1.5µg/ml tunicamycin. To decrease cytosolic calcium levels, cells were treated with 3 µM BAPTA-AM. All these compounds were obtained from Sigma (Sigma-Aldrich Chemie, Buchs, Switzerland). Anti phospho-CREB, anti-CREB, anti-phospho-eIF2alpha, anti-eIF2alpha, and anti-BiP were purchased from Cell signaling technology (BioConcept, Allschwil, Switzerland). Anti-PP2Ac was purchased from Upstate (LucernaChem, Luzern, Switzerland).

U-2 OS human osteosarcoma-derived, tetracycline (tet)-regulated cell lines UHCV-57.3, which inducibly express the entire HCV polyprotein derived from an HCV H consensus cDNA (29), and the parental cell line UTA6 transfected only with the tetracycline transactivator (tTA), and UGFP-20 cells which inducibly express green fluorescent protein, were a gift from Darius Moradpour and have been described previously (30). Human hepatoma cells Huh7 were grown in Dulbecco`s minimal essential medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. H7TA-61 (a gift of Darius Moradpour) are Huh7 cells that constitutively express the tetracycline-controlled transactivator, tTA (31). Huh7.93 cells (a gift from Michael Nassal) are derived from H7TA-61 after transfection with the HBV expressing plasmid pTRE-HBVT.

Preparation of cell extracts and immunoblotting
Whole cell and nuclear extracts were prepared as described (8). Protein concentration was determined with the BioRad Protein Assay (Bio-Rad Laboratories AG, Reinach, Switzerland). Immunoblotting was performed as described (8).

RNA isolation, reverse transcription, and XBP-1 PCR
Total RNA was isolated from cells using a Perfect RNA Eukaryotic Mini Kit (Eppendorf, Vaudaux-Eppendorf, Basel, Switzerland) according to the manufacturer`s instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega, Promega Biosciences Inc., Wallisellen, Switzerlend) in the presence of random hexamers (Promega) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70°C and then for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. To amplify XBP1 mRNA, a PCR reaction with the cDNA was performed for 30 cycles (94° for 30 s; 58°C for 30 s and 72°C for 1 min (but 5 min in a last cycle step)) using 5`-CTGGAACAGCAAGTGGTAGA-3`and 5`-CTGGGTCCTTCTGGGTAGAC-3` (32). 398bp and 424bp fragments representing spliced and unspliced XBP1, respectively, were analyzed on a 2% agarose gel.

Real time RT-PCR for PP2Ac
The cDNA was used to perform SYBR-PCR based on SYBR-Green Fluorescence (SYBR-Green PCR Master Mix, Applied Biosystems, Foster City, CA). To prevent influence from genomic DNA amplification, the primers were designed across exon-intron junctions. The primers for GAPDH were 5-GCTCCTCCTGTTCGACAGTCA-3 and 5-ACCTTCCCCATGGTGTCTGA-3 and the primers for PP2Ac were 5' CCACAGCAAGTCACACATTGG 3' and 5' CAGAGCACTTGATCGCCTACAA 3'. The CT value was derived by subtracting the threshold cycle (CT) value for GAPDH, which served as an internal control, from the CT values for PP2Ac, respectively. All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems). mRNA expression level of PP2Ac was expressed as a fold increase according to the formula $2^{\Delta CT(PBS)-\Delta CT(HCV\ expression)}$.

Electrophoretic Mobility Shift Assay (EMSA)
2 µg of nuclear extract aliquots were used to perform EMSAs. Oligonucleotides were annealed to double-strand oligonucleotides and then labeled with (γ−32P) CTP using Klenow polymerase. The sequence of the used CRE-oligo was 5`-GACGCCGGCCTGACGTCACCACGCC-3' (33). The CREB complex was supershifted with antibody 86B10 from Cell Signaling Technology (BioConcept, Allschwil, Switzerland).

CREB siRNA treatment of UHCV 57.3 cells expressing HCV proteins
UHCV 57.3 cells were transfected using Lipofectamine 2000 from Invitrogen (Basel, Switzerland) according to the manufacturer`s instructions with 20 µl of a 20 µM solution of CREB small interfering RNA (siRNA) duplexes from QIAGEN (Basel, Switzerland) or with a corresponding amount of nonsilencing siRNA duplex from QIAGEN. Four hours after transfection, cells were recovered for 48 h in Dulbecco`s minimal essential medium supplemented with 10% fetal calf serum, antibiotics for selection and

tetracycline to inhibit expression of viral proteins. After 48 h, tetracycline was removed from the medium and cells were grown for additional 24h to induce the expression of viral proteins. Whole cell extracts were prepared to perform Western blot analysis.

Calcium release of the endoplasmatic reticulum

Changes in calcium concentrations of UHCV 57.3 cells were monitored using the fluorescent calcium indicator fura-2/AM (Sigma, St. Louis, MO) at a final concentration 5 µM as described (34-36). Cells were loaded with fura-2 for 30min at 37°C, and then washed once by centrifugation, counted and the same number of cells was resuspended in Ca2+-free Krebs-Ringer medium containing 0.5 mM EGTA and placed in a cuvette thermostated at 37°C. Fluorescence changes (ratio 340/380 nm) were measured in a Perkin-Elmer LS50 spectrofluorimeter equipped with a magnetic stirrer. All measurements were made in Ca2+-free Krebs-Ringer buffer containing 0.5 mM EGTA. Experiments were performed at least four times on four different days. 1 µM thapsigargin (Sigma) was added to empty the endoplasmatic reticulum.

Results

Inducible expression of HCV proteins activates multiple endoplasmatic reticulum stress pathways

We first tested if the expression of viral proteins in cells induces an ER stress response. UHCV57.3 cells contain the entire open reading frame of a HCV consensus cDNA (30). The transcription of the HCV cDNA is under the control of a tetracycline regulated transactivator (tTA). HCV protein expression is repressed when UHCV57.3 cells are cultured in tetracycline containing medium. In the absence of tetracycline, UHCV57.3 cells express all HCV proteins (30). As shown in Figure 1, the expression of HCV proteins in UHCV57.3 cells induced the upregulation of the ER chaperon BiP and the splicing of XBP-1 mRNA, demonstrating the activation of an ER stress response in these cells. Consistent with our previous data, HCV protein expression also induced the expression of PP2Ac. ER stress and PP2Ac induction were specific for HCV protein expression, because neither was found in the control cells UTA6 and UGFP. UTA6 cells are the parental cells of UHCV57.3 cells: they stably express tTA but have no HCV cDNA. UGFP cells were derived from UTA6 cells by stable transfection of a plasmid encoding green fluorescent protein (GFP). When cultured without tetracycline, UGFP cells express high amounts of GFP (30). Interestingly, the expression of HBV proteins in Huh7.93 cells also activated an ER stress response (Figure 1E). Again, the control cell line H7TA61 (the parental cell line of Huh7.93 that contains only tTA but no HBV cDNA) did not show an induction of BiP.

Figure 1

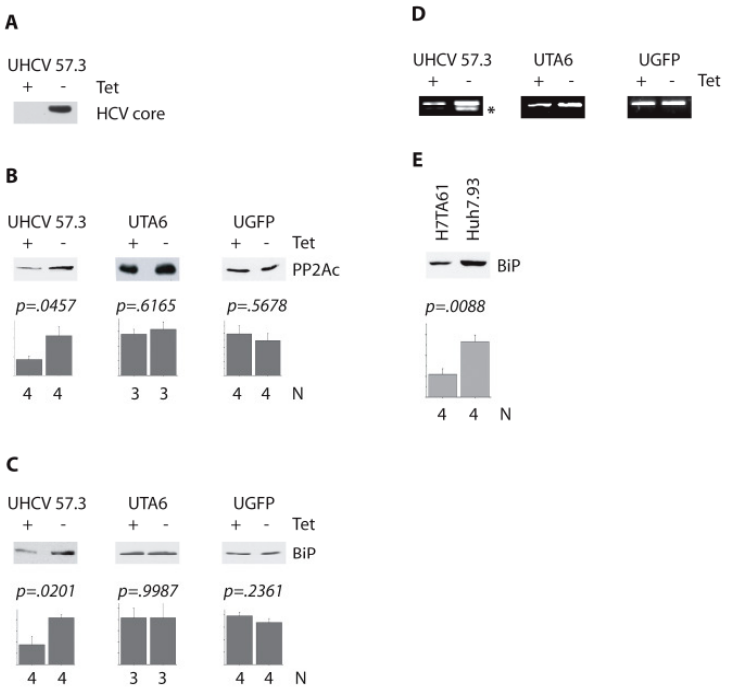

Figure 1: Expression of viral proteins leads to the induction of ER-stress markers and to an upregulation of PP2Ac. (**A**) UHCV57.3 cells express all hepatitis C virus (HCV) proteins when cultured in medium without tetracycline (HCV core protein expression is shown as an example). Control cells for HCV protein expression were UTA6 cells (contain the tetracycline transactivator tTA, but no viral transgene) and UGFP (inducible express the green fluorescent protein when cultured without tetracycline). (**B**) Expression of HCV proteins in UHCV57.3 cells upregulates PP2Ac. The upper panel shows an example of a Western blot with UHCV57.3, UTA and UGFP cells cultured in medium with or without tetracycline as indicated. The lower panel shows a statistical evaluation of densitometric measurements (arbitrary units) of the PP2Ac bands from multiple independent experiments (Number of experiments indicated below graphs). (**C**) Expression of HCV proteins in UHCV57.3 cells induces the ER-chaperone BiP. The upper panel shows an example of a Western blot. The lower panel shows a statistical evaluation of densitometric measurements (arbitrary units) of the BiP bands from multiple independent experiments (Number of experiments indicated below graphs). For all Western blot, 10µg total protein were loaded and separated on a 12% SDS-PAGE. (**D**) The activation of the IRE1-Xbp1 pathway is shown by means of XBP1 mRNA splicing (unspliced XBP1 mRNA is the upper band, and the spliced form of XBP1 is the lower band, indicated with an asterisk). No ER stress activation is observed in UTA6 and UGFP control cells. (**E**) Expression of hepatitis B virus proteins in Huh7.93 cells also induces ER stress as exemplified by the induction of BiP (example of a Western blot is shown in the upper panel). The lower panel shows a statistical evaluation of densitometric measurements (arbitrary units) of the BiP bands from multiple independent experiments (Number of experiments indicated below graphs). The control H7TA61 cells contain the tTA, but no viral cDNA.

Pharmacological induction of ER stress induces PP2Ac expression

Next we tested if pharmacological induction of ER stress could induce PP2Ac expression independent from viral protein expression. Treatment of untransfected Huh7 cells for 8 hours with thapsigargin, an inhibitor of the endoplasmatic reticulum Ca^{2+} ATPase,

induced a classical ER stress response, documented by an upregulation of BiP, a phosphorylation of P-eIF2α and splicing of the XBP1 mRNA (Figure 2A). Interestingly, PP2Ac was also strongly upregulated both at the protein (Figure 2A) and mRNA level (Figure 2B). The same upregulation of PP2Ac was observed after treatment of Huh7 cells with tunicamycin, another widely used inducer of ER stress that inhibits N-linked glycosylation of proteins in the ER and leads to the accumulation of unfolded protein (Figure 2C). To prove that pharmacological induction of ER stress could induce PP2Ac expression independent of HCV protein expression, UHCV57.3 cells were cultured in tetracycline supplemented medium to repress the expression of viral proteins, and then treated with thapsigargin for 8 hours. The treatment activated the ER stress response pathways (BiP, XBP1 mRNA splicing), and caused a strong induction of PP2Ac (Figure 2D). The same PP2Ac induction was observed in the parental cell line UTA6. We conclude that the upregulation of PP2Ac in the human hepatoma cell line Huh7 and the human osteosarcoma derived cell lines UHCV57.3 and UTA6 is an integral part of the ER stress response in these cells.

Figure 2

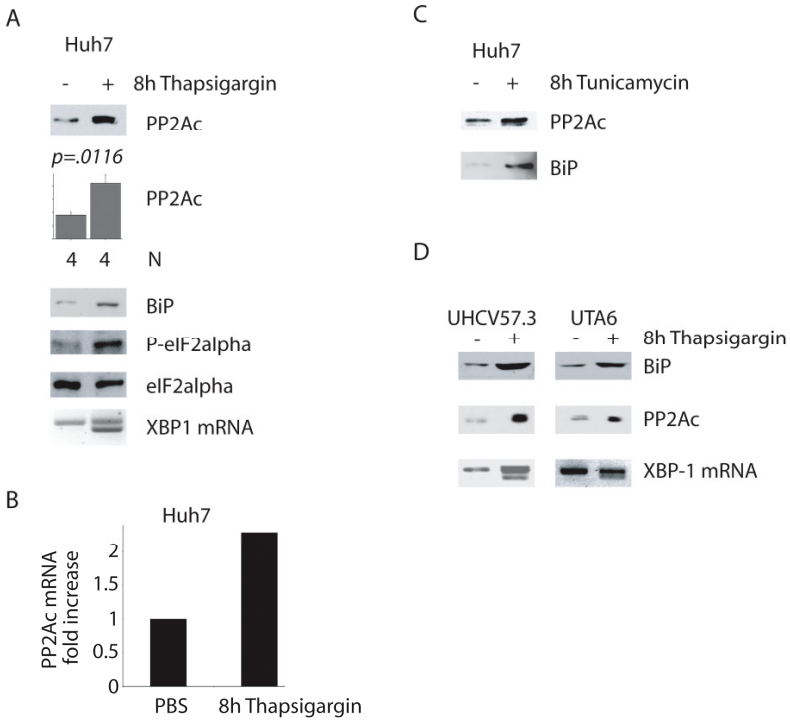

Figure 2: Pharmacological induction of ER-stress leads to an upregulation of PP2Ac that is independent from viral protein expression.
(**A**) Western blots showing the expression of BiP, PP2Ac, P-eIF2alpha and eIF2alpha and RT-PCR showing the spliced and the unspliced form of XBP1 in Huh7 cells after treatment with 1 µM of the ER-stress inducer thapsigargin for 8h. For PP2Ac induction, a statistical evaluation of densitometric

measurements (arbitrary units) of the PP2Ac bands from multiple independent experiments is shown below the Western blot example (Number of experiments indicated below graphs).
(**B**) PP2Ac mRNA concentrations in cells were measured by real-time RT-PCR. Treatment of Huh7 cells for 8h with thapsigargin resulted in a more than two-fold increase of the PP2Ac mRNA.
(**C**) Western blot showing the expression of PP2Ac and BiP in Huh7 cells after treatment with 1.5 µg/ml tunicamycin for 8h.
(**D**): Western blot showing the induction of ER-stress markers (BiP and XBP-1 splicing) and the upregulation of PP2Ac after treatment of tetracycline repressed UHCV 57.3 (left panel) and UTA-6 cells (right panel) with 1 µM thapsigargin for 8h. For all Western blot shown in figure 2, 10µg total protein were loaded per lane and separated on a 12% SDS-PAGE.

PP2Ac upregulation is mediated by CREB

The transcriptional upregulation of stress response genes is mediated through the cis-acting promoter elements ER stress response element-I (ERSE-I) and ERSE-II (37-39). We therefore scanned the promoter of PP2Ac for the presence of these sites, but did not find homologous sequences. However, a canonical CREB binding site (TCACGTCA) is present at position –241 to –233 of the PP2Ac promoter (40). Indeed, CREB was phosphorylated (activated) in UHCV57.3 cells that were induced to express viral proteins and thereby had an activated ER stress response (Figure 3A). The activation of CREB by HCV protein expression was confirmed with an electrophoretic mobility shift assay (EMSA) using a CRE oligonucleotide (Figure 3B). CREB was necessary for PP2Ac upregulation, because pretreatment of cells with a CREB specific siRNA prevented the HCV protein induced PP2Ac over-expression (Figure 3C).

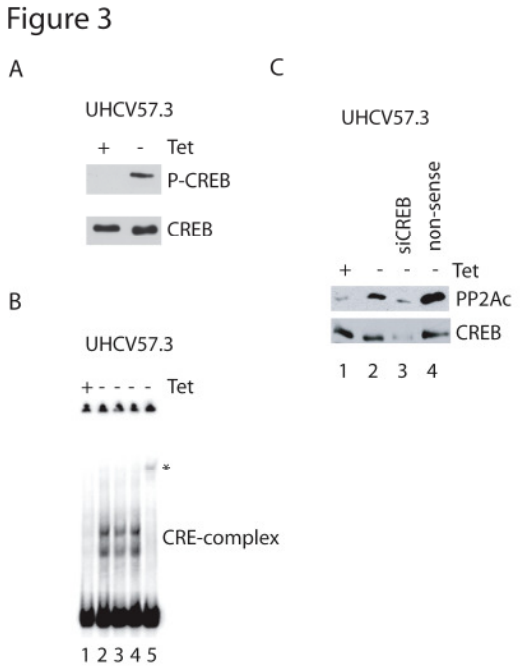

Figure 3: HCV protein expression leads to the activation of the transcription factor CREB.

(**A**) The phosphorylation of CREB was analyzed by Western blot of whole cells extracts of UHCV57.3 cells grown in the presence or in the absence of tetracycline (upper panel). Below is the loading control, showing the amount of CREB in both samples.
(**B**) Electrophoretic mobility shift assay (EMSA) with a CRE oligonucleotide and nuclear extracts of UHCV57.3 cells that have been grown in the presence of tetracycline (lane 1) or without tetracycline (lanes 2-5). The de-repression of HCV protein expression in UHCV57.3 cells induced a CREB shift (lanes 2-4) that could be supershifted (*) with a CREB specific antibody (lane5).
(**C**) Silencing of CREB prevents the overexpression of PP2Ac in UHCV57.3 cells with induced HCV protein expression. UHCV 57.3 cells were transfected with siRNA against CREB (lane 3) or with non-silencing siRNA (lane 4) for 48h. Cells were then cultured for an additional 24h in the absence of tetracycline to induce HCV protein expression and ER stress. Compared to cells grown in tetracycline containing medium (lane 1), UHCV57.3 cells cultured without tetracycline showed the expected up-regulation of PP2Ac (lane 2). This upregulation was prevented by pretreatment of cells with a CREB specific siRNA (lane 3), but not by a non-sense siRNA (lane 4). The lower panel shows the expression level of CREB in the different samples.

ER stress induced calcium release from the endoplasmatic reticulum activates CREB

CREB is an ubiquitously expressed transcription factor that can be activated by G protein coupled receptors via cyclic AMP (cAMP) and protein kinase A. Alternatively, CREB can also be phosphorylated on the same serine residue (Ser133) by members of the Ca^{2+}-calmodulin-dependent protein kinase (CaM kinase) family (41). Calcium is involved in the induction of ER stress responses (42, 43), and we therefore tested if CREB phosphorylation in response to HCV protein expression is caused by Ca^{2+}. Indeed, Huh7 cells that were treated with $CaCl_2$ showed the same amount of phosphorylated CREB as Huh7 samples treated with thapsigargin (Figure 4A). In accordance with a direct role of CREB in the upregulation of PP2Ac, $CaCl_2$ treatment of Huh7 cells also induced an over-expression of PP2Ac (Figure 4B). To test if the expression of HCV proteins caused a release of Ca^{2+} from the ER, we indirectly measured the Ca^{2+} content in the ER in UHCV57.3 cells. To this end, cells were loaded with the fluorescent calcium indicator fura-2/AM and indirect measurement of the Ca^{2+} content in the ER were obtained by treating the cells with thapsigargin, an inhibitor of endoplasmatic reticulum Ca^{2+} ATPase. The peak Ca^{2+} release induced by this treatment is an indicator of the amount of Ca^{2+} present in the ER. As shown in figure 4C, HCV protein expression in UHCV57.3 cells significantly reduced the Ca^{2+} content in the ER. Thus the expression of HCV proteins induces an ER stress response leading to a decrease of the size of the intracellular Ca^{2+} stores, via a release of Ca^{2+} from the ER. This in turn stimulates CaM kinase, leading to the phosphorylation of CREB. In support of this hypothesis, when the increase of Ca^{2+} in the cytoplasma was prevented by the calcium chelator BAPTA, HCV protein expression no longer caused an upregulation of PP2Ac (Figure 4D).

Figure 4

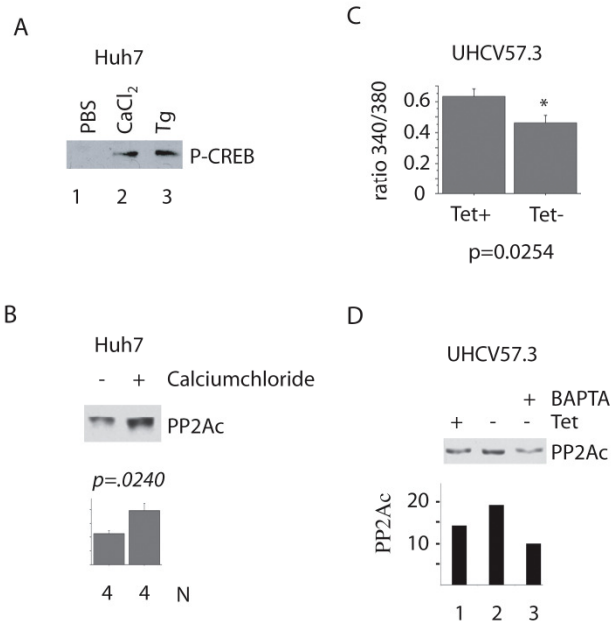

Figure 4: CREB activation and PP2Ac upregulation is mediated through Ca^{2+} signaling.
(**A**) Western blot analysis with phospho-CREB specific antibodies. Huh7 cells were treated for 16h with 1mM $CaCl_2$ or 1 µM thapsigargin. 20µg total protein were loaded per lane and separated on a 12% SDS-PAGE.
(**B**) Huh7 cells were treated with PBS or with 1 mM $CaCl_2$ for 16h as indicated and whole cells extracts were analyzed for the expression of PP2Ac by Western blot (upper panel shows an example of a Western blot). The lower panel shows a statistical evaluation of densitometric measurements (arbitrary units) of the PP2Ac bands from multiple independent experiments (Number of experiments indicated below graphs).
(**C**) Peak calcium released from the ER by thapsigargin was measured after loading the cells with the fluorescent calcium indicator fura-2/AM. The peak calcium induced by addig 1µM thapsigargin was significantly lower in cells that were grown in the absence of tetracycline to induce HCV protein expression. Results are expressed as means (+/- SE) of 16 experiments. Measurements were performed on 1.5 x 10^6 cells. Statistical analysis was done with Anova.
(**D**) UHCV 57.3 cells were grown in the presence of Tet (lane 1) or in the absence of Tet for 24h (lane 2 and 3) to induce expression of HCV proteins. One of the HCV protein expressing samples was treated with 3 µM BAPTA-AM to prevent an increase in cytosolic free Ca^{2+} (lane3). Whole cell extracts were done and PP2Ac levels were analyzed by Western blot. The lower panel shows a densitometric analysis of the Western blot signals (arbitrary units).

Discussion

PP2A is involved in a wide range of cellular processes including cell cycle regulation, cell morphology, development, signal transduction, translation, apoptosis and stress response (44). PP2A is a heterotrimeric serine/threonine phosphatase consisting of a 36 kDa catalytic C subunit (PP2Ac), a 65 kDa structural A subunit and a variable regulatory B subunit. It is a very abundant protein that accounts for an estimated 0.3% to 1.0% of

the total cellular proteins (45). Given its many functions and interactions with other cellular proteins, it is not surprising that the expression of PP2A is tightly regulated (44, 46, 47). However, downregulation of PP2Ac has been found, for instance during all-*trans*-retinoic acid-induced differentiation of HL-60 cells (48) or during peroxisome proliferator-activated receptor-γ induced adipocyte differentiation (49). Likewise, upregulation of PP2Ac was found in macrophages in response to colony-stimulating factor 1 (50), and we have previously reported that HCV protein expression in cells or in livers of transgenic mice induced the overexpression of PP2Ac (8). Here we report for the first time that PP2Ac is upregulated during ER stress, no matter if induced pharmacologically with thapsigargin or tunicamycin, or through the expression of viral proteins.

There are several potential implications of PP2A upregulation for the ER stress response. For example, ER stress is known to inhibit the cell cycle in G1 through the activation of PERK (51). Interestingly, PP2A has been shown to keep the M-phase-promoting factor (MPF, a complex consisting of the $p34^{cdc2}$ kinase (Cdc2 or Cdk1) and cyclin B) in its inactive form, preventing the G_2/M transition (52, 53). The upregulation of PP2A during ER stress might contribute to cell cycle exit by inhibiting the G_2/M transition.

Chronic ER stress results in programmed cell death through the activation of proapoptotic pathways that involve IRE1, TRAF2, caspase12, caspase3, CHOP and BCL-2 family members (19). CHOP, a proapoptotic transcription factor induced through ATF4 and ATF6 (54), suppresses the transcription of anti-apoptotic bcl-2 (55), thereby promoting cell death. PP2A has the opposite effect of enhancing the resistance to apoptosis in stressed cells. By dephosphorylating bcl-2 on serine-87, PP2A protects blc-2 from proteasome-dependent degradation (56) and enhances its anti-apoptotic function (57). The upregulation of PP2A by ER stress might therefore restrain the effects of the proapoptotic signals generated through CHOP.

The PP2Acα promoter contains neither an ERSE-I nor an ERSE-II binding site for the classical ER stress transcription factors XBP1 and ATF6. The promoter also lacks TATA and CCAAT boxes. Instead it has several Sp1 binding sites and a cAMP-regulatory element (CRE) (40). We therefore investigated if HCV protein expression leads to CREB activation, using an antibody that is specific for the Ser133 phosphorylated form of CREB (with some crossreactivity for ATF1), and found a strong induction of CREB phosphorylation and CREB DNA binding. CREB activation was required for PP2Ac induction, because CREB specific siRNA knockdown prevented PP2Ac upregulation by HCV proteins. Interestingly, PP2Ac is involved in the negative regulation of CREB. PP2A dephosphorylates Ser133 of CREB (58) and PP2A inhibits calcium/calmodulin-dependent protein kinase (CaMK) thereby preventing CREB phosphorylation (59). The HCV protein induced upregulation of PP2Ac through Ca^{2+} and CREB could therefore constitute a negative feedback loop that limits the extent of CREB mediated transcriptional regulation.

The presented findings also have implications for human chronic viral hepatitis. First, PP2Ac upregulation is not only observed cell culture systems and transgenic mice (8), but

also in liver biopsy samples from patients with chronic hepatitis C (12). Importantly, PP2Ac overexpression per se (in cells without HCV protein expression) inhibits IFNα induced intracellular signaling (8), providing an obvious advantage for viral replication and persistence. Viral interference with IFNα signaling has been described for a growing number of viruses, and in many cases a specific function of an individual viral protein that was responsible for the interference with IFNα signaling could be identified (60, 61). In the past, we have analyzed a panel of cell lines that express individual HCV protein with the aim of identify ingthe viral protein(s) responsible for the inhibition of IFNα signaling and the upregulation of PP2Ac. However, we could not unequivocally identify the responsible protein. We then discovered that the infection with hepatitis B virus also lead to an upregulation of PP2Ac in the liver. The present work provides now a unifying hypothesis how these completely unrelated viruses induce a similar cellular response that finally leads to an inhibition of IFNα signaling. Both viruses activate an ER stress response, and through the Ca^{2+} dependent activation of CREB transcriptionally upregulate PP2Ac, a key enzyme for many cellular processes. The negative regulation of IFNα signaling through PP2A is an obvious advantage for the viruses, but the role of PP2A in regulating the cell cycle and apoptotic signaling pathways might be as important.

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4. Discussion

4.1 Inhibition of the IFN Signaling by HCV

The resistance of HCV to IFNα treatment is a major health problem worldwide, and the mechanisms responsible for the treatment failure in up to 50% of treated patients are still not completely understood. A lot of effort has been made during the last years to investigate the interactions of the hepatitis C virus with the host immune system and to identify new targets for the development of a vaccine or an improved therapy. It was shown that the E2 protein of HCV interferes with the double-stranded RNA-activated protein kinase (PKR). PKR is induced by IFN and inhibits protein synthesis by phosphorylation of the translation initiation factor eIF2. HCV E2 from most HCV isolates contains a 12-amino acid sequence that is similar to the PKR autophosphorylation site and the eIF2 phosphorylation site, a target of PKR. Because of this sequence homology of E2 to PKR and eIF2 phosphorylation site, it was suggested that the E2 protein binds to and inhibits PKR in vitro and does so in mammalian cells and yeast (*113*). Another HCV protein that interacts with PKR is the nonstructural protein NS5A. NS5A repressed PKR activity through a direct interaction with the protein kinase catalytic domain. A specific amino acid sequence, the socalled interferon sensitivity-determining region (ISDR) of the HCV NS5A was required for this interaction (*114*). A third mechanism of inhibiting PKR by HCV involves the viral internal ribosome entry site (IRES). A region of the viral RNA comprising part of the IRES was able to bind to PKR in competition to double-stranded RNA and to prevent autophosphorylation and activation of the kinase *in vitro*. However the HCV IRES itself had no PKR-activating ability (*115*).
The induction of interleukin 8 (Il-8) by nonstructural protein NS5A displayed an additional mechanism to inhibit the antiviral actions of IFN. IL-8 is a 71-amino-acid chemotactic cytokine that is induced primarily by the cytokines IL-1 and tumor necrosis factor alpha (TNF-) and that is produced by many cells, including fibroblasts and hepatocytes. IL-8 is a principal mediator of the inflammatory response to many viruses and bacteria and it inhibits the antiviral actions of IFN *in vitro* (*116*).
Other important components of the innate immune system are the interferon regulatory factors (IRFs), transcription factors that initiate a cellular antiviral state (*117*). IRF-3 is a latent cytoplasmic factor that is activated through phosphorylation. Phosphorylated IRF-3 translocates to the nucleus, where it induces transcription of type I IFNs and other antiviral genes (*118*). HCV RNA replication activates IRF-3 and stimulates cellular antiviral responses (*119*). Further studies identified RIG1, a sensor-protein for double strand RNA, as the sensor of HCV RNA and inducer of IFNβ (*120*). The adaptor protein that coupled RIG1 to the downstream kinases (IKKε, TKB1) that phosphorylate IRF was CARDIF (also known as MAVS, VISA and IPS-1) (*121*). HCV NS3/4 protease cleaved and inactivated CARDIF and thereby blocked the induction of IFNβ (*121*)).
Furthermore, HCV counteracts the antiviral activity of IFN by inhibiting the Jak-STAT pathway. The work presented in this thesis contributed substantially to the current understanding of the mechanisms of HCV interference with IFNα signaling. The hallmark of this inhibition is the hypomethylation of STAT1. Reduced methylation of STAT1 was found in livers of mice expressing the whole HCV open reading frame, in

liver biopsies of CHC patients and in osteosarcoma cells expressing HCV proteins. Hypomethylated STAT1 showed an increased association with PIAS1. The association of PIAS1 to STAT1 prevented the binding of STAT1 to promoters of IFNα target genes upon IFNα stimulation and therefore leaded to a reduced induction of IFNα responsive genes. Beside the hypomethylation of STAT1, an increased level of PP2Ac was observed, that leaded to the hypothesis that PP2Ac inhibits the enzymatic activity of PRMT1 (*30, Blindenbacher, 2003 #164*). We provide the following strong evidence that this is indeed the case: first we could show elevated PP2A level in cells expressing HCV proteins, in HCV subreplicon cells and in liver biopsy samples of CHC patients. Interestingly, in biopsy samples of genotype 1 infected patients, the level of PP2A was higher than in samples of patients infected with genotype 3. This result is in accordance with the finding that genotype 1 infected patients are more difficult to treat and have a higher risk to develop chronic infection than patients with other genotypes. Second, we show that the increase expression of PP2Ac directly inhibits the enzymatic activity of PRMT1 leading to STAT1 hypomethylation. Consequently, PIAS1 associates to STAT1 and blocks the binding of activated STAT1 to the promoter of target genes. Third, the inhibitory effect of PP2Ac on PRMT1 releases NS3 helicase from arginine methylation, resulting in an increase replication of the virus.

4.2 Antiviral therapy for chronic hepatitis C

In 1989, the hepatitis C virus was discovered in the United States as causative agent of posttransfusion non-A, non-B hepatitis (Science 1989 Choo). It is estimated that 170 million people worldwide are infected with HCV (WHO). Many cases are asymptomatic and result in hepatic disease, manifested as hepatic cirrhosis or cancer. IFN therapy for hepatitis C dates from 1986, when Hoofnagle et al (1986, N Engl J Med) reported the normalization of serum alanine aminotransferase (ALT) levels following administration of recombinant human IFNα to patients with non-A, non-B hepatitis. IFN was shown to be biochemically effective as an antiinflammatory drug before the discovery of HCV in 1989 (*31*). The therapeutic outcome to IFN can be classified as sustained virologic response (SVR), relapse, or nonresponse. SVR means complete elimination of HCV, defined as the loss of detectable HCV RNA during therapy and for at least 6 months after therapy. Relapse is defined as being HCV-negative at the end of treatment but HCV-positive within 6 months after therapy. Finally nonresponse is defined as the absence of an HCV-negative period during the treatment. Unfortunately, therapy with IFNα alone had only limited success with a sustained response rate of approx. 20%. A major advance came with the addition of the broad-spectrum antiviral agent ribavirin to IFNα therapy, which more than doubled the sustained response rate to 35-40% (*122*). Further improvement has been achieved by the development of pegylated interferon, in which a large molecule of poly (ethylene glycol) (PEG) is covalently attached to recombinant IFNα, resulting in an active molecule with a longer half-life, better pharmacokinetic profile and better rate of virological response (*123*). The combination of PEG-IFNα with ribavirin yields sustained response rates up to 56%. Viral factors that correlate with a

sustained response to combination therapy are genotype, viral load and quasispecies diversity. But host factors also affect the chance of clearance of the virus, albeit less so than the genotype does. These include age, race, gender, obesity and degree of hepatic fibrosis. Among these factors, racial differences in response rates are the most striking (*124*). The reasons for the racial differences in response rates to pegylated interferon and ribavirin therapy for hepatitis C are not known. The current commercially available forms of IFNα used for for hepatitis C are α2a, α2b and consensus IFN. IFNα has potent antiviral activity but does not act directly on the virus or replication complex. Rather, it acts by inducing IFN-stimulated genes (ISG), which establish a non-virus-specific antiviral state within the cell (*125*). Microarray analysis show that hundreds of genes are induced by type-1 IFN via the Jak-STAT signaling pathway, many of these genes related to antiviral activity but others involved in lipid metabolism, apoptosis, protein degradation and inflammatory cell responses (*126*).

Because of the low efficiency and the adverse side effects of IFNα-ribavirin combination therapy, new treatment regimens are needed. Major research efforts have concentrated on the identification of agents that inhibit specific steps in the life cycle of the virus. A straightforward approach was the development of antiviral drugs that blocks essential viral enzymes. Although all HCV enzymes are good targets for therapeutic intervention, the NS3-4A serine protease and the NS5B RNA polymerase have emerged as the most popular targets. A number of competitive inhibitors of NS3 protease as well as nucleoside and non-nucleoside inhibitors of the NS5B polymerase have been developed. Some of theses compounds are already used in early-phase clinical trials. But the problem of these compounds is the development of drug-resistant viruses under the selective pressures exerted by antiviral drugs. The fast turnover rate and the intrinsic low fidelity of the HCV replication machinery endows the virus with the ability to fully explore its genome space and quickly come up with mutations that render it resistant to antiviral drugs. Another interesting approach to develop new drugs against HCV is the use of RNA interference (siRNA). In tissue culture, siRNA, as well as vector-encoded short hairpin RNA (shRNA) directed against the viral genome, effectively blocks the replication of HCV replicons, the most effective siRNA are capable of completely eradicating HCV from more than 98% of the replicon bearing cells (*127*). Despite the positive outcome of the *in vitro* studies, there is the problem of efficient *in vivo* delivery of siRNA. Chemically modified siRNA seem able to inhibit HCV replication in tissue culture and also in a mouse model of HCV infection (Han, Symposium Heidelberg 2004).

A completely other approach is the development and use of immunomodulatory agents. The experience with the IFN therapy has demonstrated, that HCV infection can be eradicated by agents that stimulate the host immune system. Following this line, synthetic agonists of Toll-like receptors (TLRs) 7 and 9 have progressed through early-phase clinical trials. TLR are molecules that sense the presence of invading microorganisms and initiate acute inflammatory responses by induction of antimicrobial genes and pro-inflammatory cytokines and chemokines. For example potent agonosts of TLR-9 are short synthetic oligonucleotides containing one or more unmethylated CpG motifs flanked by specific sequences.

In the present work, we show another approach to improve the IFNα therapy by overcoming the inhibition of the Jak-STAT signaling through the hepatitis C virus. As described above, the inhibition of the signaling is caused by the decreased activity of

PRMT1 that cause an accumulation of hypomethylated STAT1, and as a consequence increased association of STAT1 with PIAS1 and therefore less IFNα target gene induction. For the methylation of STAT1, PRMT1 uses S-adenosylmethionine (AdoMet, SAMe) as a methylgroup donor. The products of this reaction are methylated STAT1 and S-adenosylhomocysteine that is converted via homocysteine into methionine. As last step of this biochemical cycle, methionine is transformed into AdoMet. Manipulation of the cycle through increasing the AdoMet concentration in a cell can stimulate the activity of PRMT1. Increased activity of PRMT1 would lead to more methylation of PRMT1 substrates but also to an accumulation of homocysteine, which is toxic for cells. Therefore the rate of transformation of homocysteine into methionine has to be increased by administrating Betaine to the cells. Betaine is the principal methyl donor for the generation of methionine from homocysteine.We provide evidence that AdoMet and betaine can correct HCV induced STAT1 hypomethylation. First, treatment of cells that express a constitutive active HA-PP2Ac and therefore have hypomethylated STAT1, with AdoMet and betaine increases the methylation of STAT1. Second, the administration of AdoMet and betaine to cells expressing HCV proteins prior to the stimulation with IFNα restores the Jak-STAT signaling. And third, treatment of Huh7 cells haboring a subgenomic HCV replicon, a well established model for HCV replication, with IFNα inhibits HCV subgenomic RNA replication. By supplementing IFNα with AdoMet and betaine, we could increase the effect of the IFNα about tenfold. Taken together, our results suggest the addition of AdoMet and betaine, two nontoxic substances that are available in many countries without prescription, to the current standard treatment of CHC with pegIFNα and ribavirin could increase the efficacy of the treatment. This hypothesis has to be tested in clinical trials.

4.3 Induction of ER stress and the consequences for the cell

The unfolded protein response (UPR) deals with adverse effects of ER stress in a timely and efficient manner at the early stage and thus enhances cell survival. But prolonged ER stress, induced for example by viral infection, has severe consequences for the cell, including apoptosis and disturbed lipid metabolism. For example, to resolve ER stress, sustained UPR consumes energy in retrotranslocating unfolded or misfolded proteins retained in the ER to the cytoplasm for ubiquitination (*128*). This energy depletion can contribute to programmed cell death (*129*). The activation of JNK and mitochondria-dependent caspases during ER stress leads also to apoptosis (*130*). Another death-signaling pathway activated by ER stress is mediated by transcriptional activation of CHOP, that potentiates apoptosis through repressing expression of anti-apoptotic Bcl2 and Bcl-X_L, and induction of ER oxidase 1α which generates reactive oxygen species and depletes reduced glutathione (*103*). In addition, prolonged ER stress is associated with release of ER Ca^{2+} stores, which can perturb mitochondria, triggering oxidative stress. Ca^{2+}-induced oxidative stress can induce both cell death and activate NF-κB signaling, contributing to inflammation (*131*). Aside from cell death and inflammation, ER stress

contributes to intracellular lipid accumulation, which is mediated by the ER-associated transmembrane sterolresponse element-binding proteins (SREBP) (*132*). Once activated during ER stress, SREBP acts as transcription factors of fatty acids/triglycerides and cholesterol, respectively, and cellular uptake of lipoproteins (*133*). ER stress-induced overproduction of lipids can lead to fatty liver (*134*).

The UPR triggered by the translation of viral proteins can be viewed as a two edged sword. On the one hand, the UPR can promote cell survival, which impairs viral eradication. On the other hand, the UPR-induced PERK-mediated translation inhibition could suppress viral protein synthesis. It is known that HCV induces components of the UPR, a variety of evidence indicates that individual HCV proteins modulate the UPR and ER stress response which can result mainly in increased viral replication and failure to eliminate infected cells. For instance, NS4B induces ATF6 and IRE1 to favor the HCV subreplicon and HCV viral replication (*135*). The expression of the HCV genes correlates with the translocation of the cytoplasmic domain of ATF6 to the nucleus of cells harboring HCV subgenomic replicons. Beside of the activation of ATF6, XBP1 spliced mRNA and sXBP1 protein levels are elevated in HCV replicon-expressing cells (*111*), but the transactivation activity of sXBP1 is repressed in the HCV-infected cells, which prevents the induction of the ER degradation-enhancing-mannosidase-like protein (EDEM) (*136*). HCV infection also modulates protein synthesis through PERK by suppressing PERK activity through HCV E2 (*112*). In addition, HCV induced ER stress results in reduced protein glycosylation, which disrupts the proper folding and assembly of MHC class 1 molecules (*137*). Therefore cells expressing HCV subgenomic replicons have lower MHC class 1 cell surface expression and less antigen presentation to cytotoxic T lymphocytes. This ER stress-mediated interference of MHC class1 assembly and cell surface expression may contribute to the persistence and pathogenesis of HCV infection. But it remains to be investigated if these observations reflect of what occurs in vivo in HCV-infected patients and if a attenuated UPR contributes to the chronicity of viral infection or if the HCV-induced ER stress response promotes the progression to apoptosis and inflammation.

In the present work, we could show that expression of HCV proteins in human osteosarcoma cells induce an ER stress response. One result of prolonged ER stress is the release of ER Ca^{2+} stores into the cytosol. Therefore the cytosolic calcium-concentration increases for a short time, before the Ca^{2+} enters other calcium-storage compartments like the mitochondria. But these short calcium-transients are enough to activate Ca^{2+} -dependent kinases, that phosphorylate the transcription factor CREB. Until now, we could not identify the kinase responsible for the phosphorylation of CREB, but one good candidate kinase is the Calcium-calmodulin-dependent kinase II, which is known to be expressed ubiquitous and which can phosphorylate CREB in a Ca^{2+-} dependent manner. However, we could demonstrate with siRNA experiments that CREB is responsible for the PP2Ac up regulation. The activated CREB binds to the PP2Ac promoter and activates PP2Ac transcription.

4.4 Summary

Summarizing all our results, we can draw the following picture (Fig.6). The expression of HCV proteins induces an ER stress response. The consequence of prolonged ER stress is the release of Ca^{2+} from the ER into the cytosol. Cytosolic Ca^{2+}-transients activate Ca^{2+} dependent kinases, which phosphorylate the transcription factor CREB. Phosphorylated CREB binds to the CRE-element in the PP2Ac promoter and induces transcription. Elevated PP2Ac level inhibits the enzymatic activity of PRMT1. Two consequences of reduced PRMT1 activity are the hypomethylation of STAT 1 and the hypomethylation of NS3 helicase. Hypomethylated STAT1 shows increased association with PIAS1 and decreased binding to IFNα-target genes upon IFNα treatment. The consequence of the inhibited IFNα-signaling is a reduced antiviral response, which contributes to the development of chronic liver infection, which later on can progress to liver cirrhosis and HCC. On the other hand, the unwinding activity of hypomethylated NS3 is increased compared to methylated NS3. The higher unwinding activty of NS3 causes an increase in viral replication. Therefore the upregulation of PP2Ac has two advantages for the virus: reduced cellular antiviral response and increased viral replication.

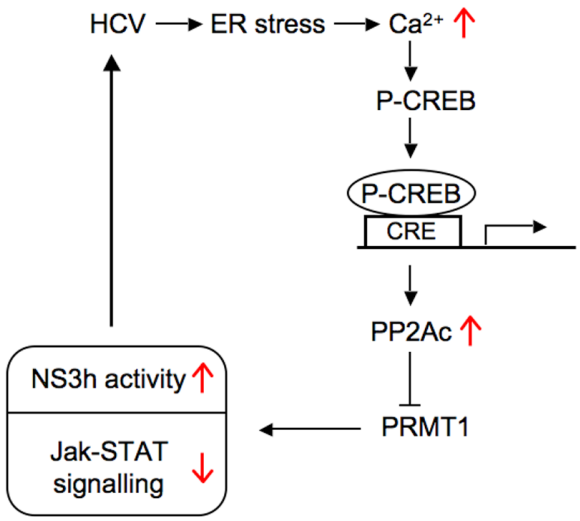

Fig.6: The consequences of the ER stress induced by HCV.
The expression of HCV proteins induces ER stress, increased Ca^{2+} concentrations in the cytosol, phosphorylation of CREB and increased PP2Ac transcription. PP2Ac afterwards inhibits the enzymatic activity of PRMT1. Two consequences of decreased PRMT1 activity are the inhibition of Jak-STAT signaling and the increased activity of NS3 helicase resulting in more HCV replication.

5. Perspectives

The promising results of the improved IFNα signaling in UHCV 57.3 cells (express HCV proteins in the absence of tetracycline) after AdoMet/Betaine treatment and the fact that the expression of HBV proteins in Huh7.93 cells induces obviously the same IFNα inhibiting mechanism (increased PP2Ac levels, hypomethylated STAT1 and decreased induction of IFNα target genes), it opens the interesting perspective of correcting the HBV induced defect in IFNα signaling also by treating cells with AdoMet and betaine. We would expect, that the AdoMet/betaine treatment would at least partially restore the IFNα signaling in Huh7.93 cells and would lead to an increased induction of antiviral genes compared to Huh7.93 cells not treated with AdoMet/Betaine.

AdoMet and Betaine are two compounds, which are already on the market as over the counter drugs, and which are used to treat liver diseases. Now that we could show improved IFNα signaling in UHCV57.3 cell pretreated with AdoMet and betaine, we plan to apply these drugs to HCV patients in a clinical study. The plan is to retreat non-responders with a combination of AdoMet, Betaine, IFNα and rabavirin.

To increase the activity of PRMT1 by manipulating the methionine/AdoMet cycle is one possibility to correct the inhibition of the IFNα signaling by HCV. Another possibility would be to target the first event, induction of ER stress by HCV. Without the induction of ER stress during expression of HCV proteins, Ca^{2+} would not leak out of the ER and the phosphorylation of CREB would not occur, so the level of PP2A would not rise and therefore there would be no inhibition of PRMT1. One possibility to avoid ER stress is the treatment of cells with chemical chaperones. Chemical chaperones are a group of low-molecular-weight componds, which can reverse the mislocalization and/or aggregation of proteins(*138*). Polyos such as glycerol, trimethylamines such as trimethlyamine N-oxide (TMAO), amino acid derivatives and other compounds like 4-phenylbutyric acid (PBA) and membrane-permeable forms of enzyme antagonists belong to the chemical chaperones. The mechanisms by which chemical chaperones function are not fully understood but are thought to include stabilization of improperly folded proteins, reduction of aggregation, prevention of nonproductive interactions with other resident proteins and alteration of the activity of endogenous chaperones in such a way that the affected proteins are more efficiently transported to the appropriate intracellular or extracellular destination.
An interesting experiment would be the expression of viral proteins in UHCV57.3 cells for 24h in the presence of chemical chaperones, and check afterwards for the level of PP2Ac and for the induction of ER stress markers like BiP or spliced XBP1. We would expect, that the increased level of chaperones in the ER prevent the induction of ER-stress and the Ca^{2+} -transients in the cytosol. Therefore the transcription factor CREB does not get phosphorylated, and the PP2Ac level stays constant even in the presence of viral proteins.

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Publication list:

Activation of endoplasmatic reticulum stress response upregulates protein phosphatase 2A
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